Mechanisms of Host Resistance to Infectious Agents, Tumors, and Allografts

A Conference in Recognition of the Trudeau Institute Centennial

Edited by Ralph M. Steinman and Robert J. North

The first tuberculosis "cure cottage" in North America at Saranac Lake, New York

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Preface

In July 1985 a scientific meeting was held at Saranac Lake, New York, to complete the year-long celebration of the centennial of the Trudeau Institute. This text is a compilation of the contributed papers.

In mid-1884 Dr. Edward Livingston Trudeau built the first cure cottage of the Adirondack Cottage Sanatorium for the Treatment of Tuberculosis at Saranac Lake. It is a view of that first cottage, preserved today as a monument to Dr. Trudeau's vision of improved health for victims of tuberculosis, that faces the title page of this book. Declining patient census brought about the closing of the sanatorium in 1954. Funds derived from the consolidation of the sanatorium properties and assets were used to establish the Trudeau Institute, a research facility now devoted to the study of host resistance mechanisms.

The grandson of Edward Livingston Trudeau, Francis B. Trudeau, believed that the placement of the Institute in the Adirondack Mountains would be conducive to the creative thinking necessary for thoughtful and independent research. This charge was fulfilled with remarkable speed. Under the leadership of Dr. George Mackaness, the first director, Trudeau investigators developed methods and concepts still pertinent to the analysis of mechanisms of host resistance. Using models of rodent tuberculosis and listeriosis, Institute scientists identified the respective functions of T lymphocytes and activated macrophages as antigen-specific and nonspecific mediators of immunity. A constant emphasis of the research was its demand that immunity be measured in the whole animal, and that the meaning of any assay or measurement be interpreted in light of in vivo function. This emphasis persists in all of the Institute's broadened scientific activities, but is most notably illustrated by the development of systems in which effector and suppressor T cells are evaluated in terms of their influence on established tumors.

In keeping with the research mission of the Trudeau Institute, the July meeting dealt with our understanding of the immune response to infectious agents, tumors, and allografts. Its major purpose was to provide for interactions among researchers actively engaged in the study of immunology at cellular, molecular, and physiologic levels. Although knowledge of the ingredients of the immune response has expanded enormously, there are still few modalities to manipulate the immune system in situ, and there still is insufficient understanding of why the immune system fails to protect against a number of important replicating agents, be they

infectious or cancerous. As is evident in this volume, the meeting participants considered the composition and function of lymphoid and mononuclear phagocyte systems, and the response of the host to several important antigens as measured in vitro and in situ. The lymphocyte cell surface and the function of cytokines were the main molecular subjects of the meeting.

Readers of this volume will quickly appreciate that the Trudeau meeting by no means covered all of the issues and unknowns in cell-mediated immunity. Nonetheless, the individuals involved represent a unique blend of investigators. Their topics and experimental approaches are important and timely, and they clearly have considered their findings in the context of whole animal physiology and pathology. Our primary aim in publishing this compendium was to reach the young investigator who might otherwise find it difficult to obtain a current, succinct overview of the immune system as it relates to mechanisms of host resistance.

We are grateful to the contributors, both for their active participation in the sessions and for providing manuscripts. We hope that their contributions reflect our unifying concern of gaining an understanding of the immune system and our desire to use the knowledge so obtained to improve health, much as was hoped for in the efforts to improve resistance to tuberculosis 100 years ago.

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Lymphocytes

Structures of Marker Antigens of T Lymphocytes

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Summary

One point of particular interest arising from studies on the structure of T marker antigens is that many of these molecules are related in evolution to the immunoglobulins. Other results highlight differences among molecules, including the diversity in their carbohydrate structures and also in their mode of attachment to the lipid bilayer. The structural data provide a new framework within which functional possibilities can be explored.

Background

Specific antibodies against lymphocyte marker antigens were first produced when antisera were raised by immunizing with thymocytes or leukemia cells across mouse strains (allogeneic immunization) (1, 2). Important marker antigens identified in this way include Thy-1, Lyt-1 (now called CD5, see Table I), Lyt-2, and Lyt-3 (CD8). The Thy-1 antigen was the first marker to distinguish T lymphocytes from B lymphocytes (3) and the Lyt-1, -2, and -3 antigens were used to show that T lymphocytes could be split into the T helper (henceforth called T_{CD4})[1] and T cytotoxic/suppressor (T_{CD8}) categories (4). The initial description of subsets was based on antibody-dependent complement killing of cells, but with fluorescence-activated cell sorter analysis it was found that only the Lyt-2 and -3 antigens were truly specific for a subset of cells (5, 6). The Lyt-1 antigen was found to be on all T cells but the amount of antigen was highest on Lyt-2, 3-negative cells, and these were preferentially killed in cytotoxicity assays. An antigen that was restricted to the T_{CD4} subset

1. T_{CD4} and T_{CD8} are abbreviations for T subsets labeled by monoclonal antibodies for the CD4 and CD8 antigens, respectively. These subsets have been previously termed T_{helper} and $T_{cytotoxic/suppressor}$ cells, but this is misleading because the T_{CD4} subset can include cytotoxic cells.

Table I. Well-characterized Surface Molecules That Can Be Used as Markers for Thymocytes and Unactivated T Lymphocytes

Molecules	*Apparent molecular weight*	*Commonly used names*			*Ig-related*	*Expression*	*Comments*
		Humans	*Mice*	*Rats*			
	Mr × 10^{-3}						
T receptor complex							
T antigen receptors (Tcr)	gp43: gp40	No antibodies react with all Tcr			Yes, both chains	Thymocytes, T helper, and T cytotoxic cells	Tcr seems same for T helper and T cytotoxic cells (13)
CD3	gp25: gp20: p20	T3, Leu-4	N.A.	N.A.	Not p20; gp25 and gp20 not known	Thymocytes and mature T cells only	Associated with Tcr: antibodies mitogenic if cross-linked at surface (14)
Pan T antigens							
Thy-1	gp18	Thy-1	θ, Thy-1	Thy-1	Yes	Neuronal and fibroblasts in all species studied, thymocytes in mice and rats, T cells in mice but not rats or humans	Anti-Thy-1 mitogenic for mouse T cells. Thy-1 has a glycophospholipid hydrophobic domain
CD2	gp50	T11, LFA-2	N.A.	N.A.	Not known	Thymocytes, T lymphocytes	Receptor for sheep erythrocytes. Antibodies can be mitogenic (15)

CD5	gp67	T1, Leu-1	Lyt-1	OX-19	Not known	Thymocytes, T lymphocytes, B lymphocyte subset	Antibodies augment T lymphocyte responses
LSGP	gp95	L10	N.A.	W3/13	No	Thymocytes, T cells, neutrophils, plasma cells, brain	Mucinlike, peanut lectin receptor, deficiency may cause Wiskott–Aldrich syndrome
T subset markers							
CD4	gp55	T4, Leu-3	L3T4	W3/25	Yes	Thymocytes, T helpers, macrophages	Antibodies block T helper response to antigen
CD8	gp38: gp34 or gp34 × 2	T8, Leu-2	Lyt-2,3	OX-8	Yes	Thymocytes, T cytotoxic, NK cells	Antibodies block T cytotoxic cell reactions. Antigen is homodimer in humans, heterodimer in rodents
L-CA	gp180–240	L-CA	T200, Ly-5	L-CA	No	All leukocytes but not erythroid cells. Absent from other tissues. Determinants can distinguish among lymphocytes	Sequence shows 80,000 M_r cytoplasmic domain

Table I. Well-characterized Surface Molecules That Can Be Used as Markers for Thymocytes and Unactivated T Lymphocytes (continued)

Molecules	*Apparent molecular weight*	*Commonly used names*			*Ig-related*	*Expression*	*Comments*
		Humans	*Mice*	*Rats*			
Markers relevant to thymocytes							
MHC Class I	gp43: p12	HLA A,B,C	H-2 K,D	RT1A	Yes	Many cells, not brain nor 70% of thymocytes	Recognized along with foreign antigen by Tcr
TL (CD1?)	gp43: p12	NA1/34 T6	TL	N.A.	Yes	Thymocytes and leukemias	Similar to and genetically linked to MHC class I (16)
MRC OX-2	gp47	N.A.	N.A.	OX-2	Yes	Thymocytes, B lymphocytes, neuronal, endothelium, follicular dendritic	Appears before Thy-1 in brain development

Names for antigens are traditional or, where possible, the new nomenclature for human antigens (12) is used. In the following references, many of the data are described for humans (12), mice (17), and rats (18). Also, see Reference (19) and references in legends to Figures 1 and 3. Equivalent human, mouse, and rat antigens are grouped where the similarities seem strong but this has been proven by sequence for only Thy-1, CD8, MHC class I, and Tcr. N.A., no antibody but a mouse equivalent of CD3 has been identified at the cDNA level (14) as has a human equivalent of MRC OX-2 (G. McCaughan, M.J. Clark, and A.N. Barclay, unpublished observation). gp, glycoprotein; p, protein; apparent molecular weight, $M_r \times 10^{-3}$.

was not identified until the monoclonal antibody (MAb) approach was used—first in rats (7), then in humans (8) and mice (9).

Problems with the allogeneic approach to identifying antigens were: (*a*) a molecule would only be immunogeneic if by chance its structure differed among the strains used; (*b*) the method could only be routinely used in animals where inbred strains were available; and (*c*) antisera obtained were often of low titer and sometimes low affinity and might also contain antibodies against more than one antigen.

The approach of immunizing between species (xenogeneic) had the advantages that (*a*) most molecules would be immunogeneic from having diverged between the species, (*b*) high-affinity antibodies could be raised, and (*c*) any species could be used. The major drawback was that any serum would contain multiple specificities and it was difficult to produce an assay that was specific for only one molecule (10).

The use of the MAb technique solved the problem of xenogeneic immunization (11) and opened the way to the rapid detection of lymphocyte surface antigens in any species. This has been particularly applied in humans and there are now more marker antigens defined for human lymphocytes than for any other species (12). The features of the most well-defined T marker antigens are summarized in Table I.

The use of MAbs has been an important factor in the detailed structural analyses of all T cell surface molecules except the class I major histocompatibility complex (MHC) antigens and Thy-1. Antigens can be purified with MAb affinity chromatography and then their protein and carbohydrate structures can be analyzed (20). From the purified protein, peptide sequences can be determined and used to construct oligonucleotide probes suitable for screening a complementary DNA (cDNA) library that includes sequences coding for the antigen of interest (21). The full sequence is then obtained at the nucleic acid level. Another approach that has been very successful with T marker antigens involves transfecting DNA into a cell that does not express the antigen in question and detecting cells that have been transformed to express the antigen (22, 23). From messenger RNA (mRNA) in these cells, cDNA is copied and the sequences that differ between the antigen-positive and untransfected cells are isolated. Ultimately the identity of the correct cDNA is proven by showing that antigen expression is achieved by transfection with the isolated sequence.

Patterns of Antigen Expression

A comparison of known surface molecules of T and B cells reveals few molecules that are identical on both cell types. In contrast, among mature

T cells it seems that most surface molecules are the same (19). Thus there are a variety of antibodies that label all T cells but not B cells (Table I), but the split of T cells into the much-studied T_{CD4} and T_{CD8} subsets is thus far marked only by the CD4 and CD8 antigens themselves. The T_{CD4} subset can be further subdivided on the basis of at least two antigens (24, 25) to give two categories of cells that mediate different functions (see Chapter 6). Most lymphoid cells in the thymus (the so-called cortical cells) are believed to be dead-end cells and their surface antigen phenotype is very different from that of mature B or T cells (19). This fits with the idea that these cells constitute an alternative (abortive) endpoint in differentiation to the cells that leave the thymus as immunocompetent T lymphocytes (26).

Although many cell surface molecules show differences in expression among the various lymphocyte types, most of these are not specific to any one cell type or lineage. For example, the CD4 and CD8 antigens that are widely used to subdivide T lymphocytes are also expressed respectively on macrophages (CD4) (27) and the natural killer (NK) cells (CD8) that have not rearranged their T cell receptor genes (28). It seems possible that the only set of molecules that is quite T lineage-specific may include those involved in the T lymphocyte receptor complex, namely, T lymphocyte antigen receptor (Tcr) α- and β-chains and CD3 γ-, δ-, ε-chains. A practical consequence of this is that no single specificity among the other marker antigens can be used to identify a cell type.

Basis of the Antigenicity

All T marker antigens thus far analyzed are glycoproteins and thus the antigenic determinants could be protein- or carbohydrate-based. Current evidence suggests that usually the antigenicity is determined by the protein. For the mouse Thy-1.1 and Thy-1.2 alloantigens, a difference of a single amino acid at residue 89 correlates with the serologic allotypes (29). It should also be noted that, thus far in all cases where a T marker antibody reacts with cell surface molecules of unrelated cell types, the antigenicity appears to be due to expression of the same glycoprotein. This includes Thy-1 (29), OX-2 (21), CD5 (Ly-1) (30), and CD4 (27) antigens. In the case of Thy-1 and OX-2, the protein part for the thymus and brain forms is the same but the carbohydrate structures differ.

Some Structural Features of the Marker Antigens

One major theme emerging from structural studies on marker antigens is that a number of these molecules are related to each other and to the

immunoglobulins (Igs) in evolution. Other points that will be discussed are (*a*) diversity in carbohydrate structures, (*b*) molecular heterogeneity in the leukocyte-common antigen (L-CA) that gives rise to a T subset marker, and (*c*) diversity in the manner of membrane integration. In discussing these points emphasis will be on antigens other than the Tcr–CD3 complex.

Marker Antigens in the Immunoglobulin Superfamily

The best-known T cell marker antigens are all in the Ig superfamily and their structural relationships are illustrated in Figure 1 in comparison with a structure for the Tcr molecule. In the figure, segments that are Ig-related are shown as circles coded as being related to either Ig V or Ig C domains. A V-related structure does not imply variability as is seen in Ig and Tcr V domains, but rather that the molecule is likely to have a folding pattern like the V or C domain of Igs. The features of the Ig fold and the reasons for making the assignments shown in the figure have been extensively discussed elsewhere (29, 35). The sort of relationships seen are illustrated in Figure 2, which shows alignments of the V-domains of an Ig κ-chain and a Tcr α-chain and the V-like segments of CD8, Thy-1, and OX-2 antigens. The V-like segment of CD4 (not shown) also shows the conserved patterns of sequence seen with the sequences in Figure 2 (33).

The largest of the T cell molecules in Figure 1 is only about one-third of the size of Ig and this is possibly because the T cell molecules function only as recognition and triggering molecules at the cell surface. Immunoglobulin also mediates a cell surface receptor function on B lymphocytes but, in addition, in the secreted form, it interacts with other molecules (e.g., complement components) via the Fc part.

It can be argued that all the Ig-related domains are likely to be involved in recognition phenomena because all the known functions for Ig or Ig-related molecules involve an interaction with another molecule to trigger a subsequent event in solution or at the cell surface. In extensive studies on antibodies, no enzymatic activities have been identified but these have been observed to reside in molecules whose function is triggered by antibodies, for example, the serine proteases of the complement cascade.

Among the T cell molecules, functions are known only for the Tcr and MHC antigens; Tcr is the antigen receptor and foreign antigen is recognized on presenting cells along with MHC antigens. Antibodies against CD4, CD8, and Thy-1 can have functional effects on T cells but it is not clear how this relates to the physiologic role of the molecule. Antibodies to CD4 and CD8 can block the response of T_{CD4} and T_{CD8} cells to antigen stimulation, and it has been postulated that the CD4 and CD8

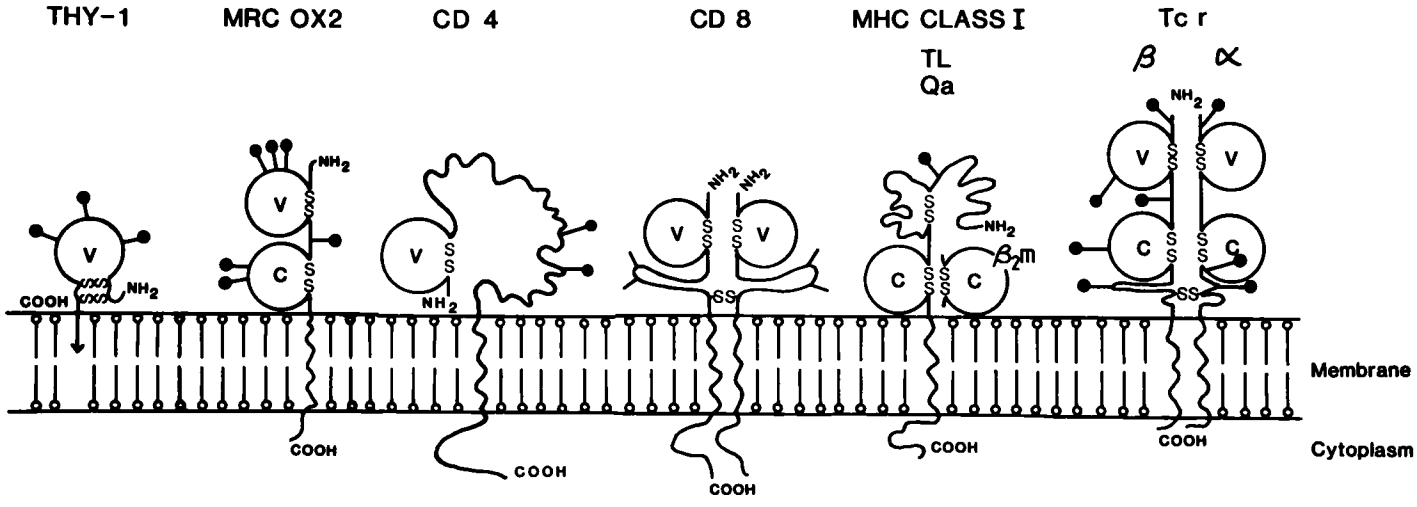
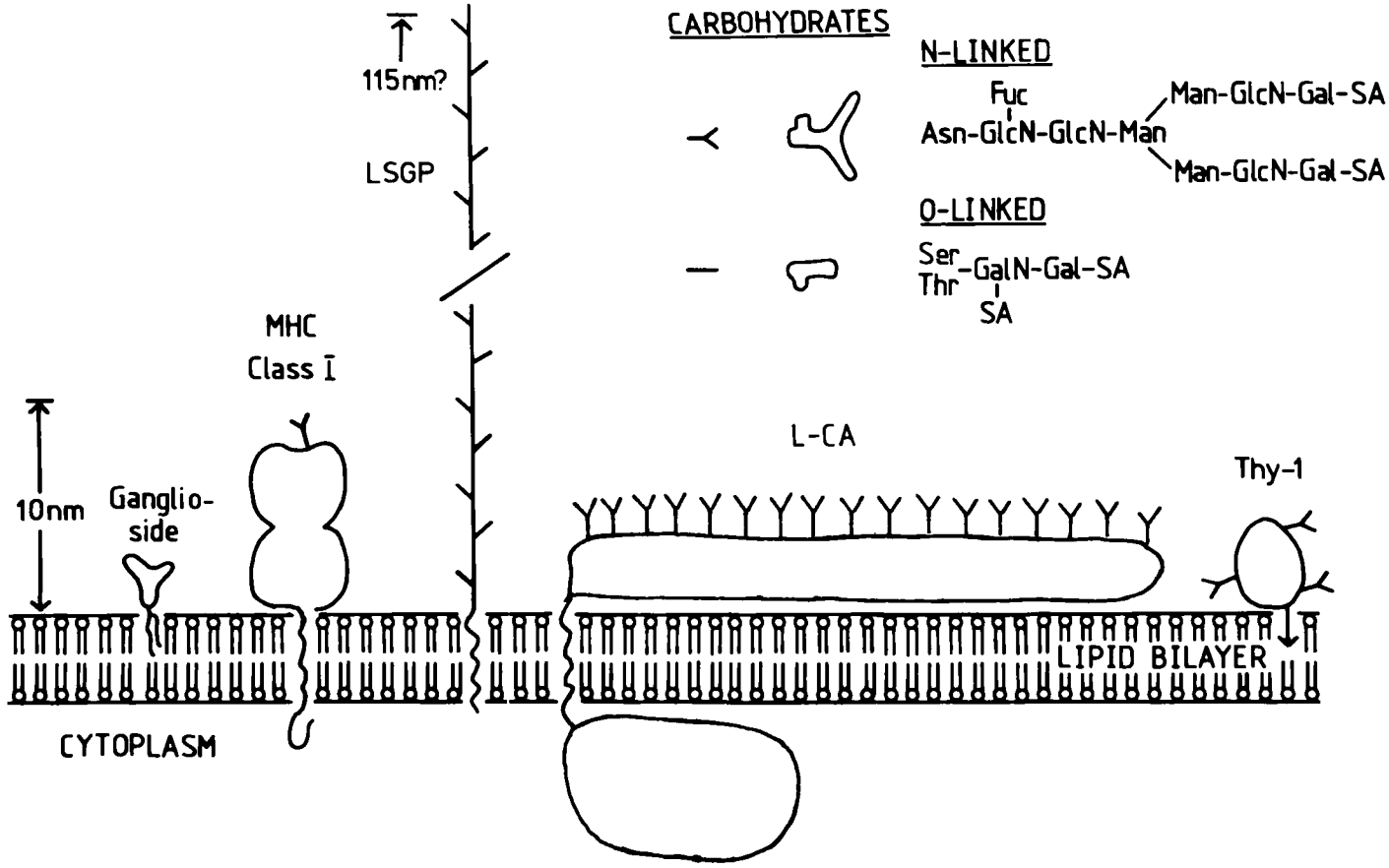


Figure 1. Models for Ig-related molecules found on thymocytes and/or unactivated T lymphocytes. Circles indicate segments related to Ig domains with similarity to a V domain (V) or C domain (C) folding pattern. Double S symbols indicate possible positions of disulphide bonds but these are established only for Thy-1 and MHC class I antigens and not all Cys residues are indicated. *N*-linked carbohydrate structures (🍴) are determined by positions of Asn X Thr or Ser in the protein sequence but the number of *O*-linked structures (|) in CD8 is arbitrary and the positions are guesswork. The model for CD8 is shown as a homodimer as for the human antigen (23); in mice (31) and rats (32), the antigen is a heterodimer one chain of which has been sequenced in each case. References for other molecules are Thy-1 (29), OX-2 (21), CD4 (33), MHC class I, TL, Qa (16, 34), Tcr (13).

Figure 2. Alignments of human CD8 (23), rat MRC OX-2 domain I (21), rat Thy-1 (29), V domain of Tcr β-chain 86T$_1$ (13) and V κ J606 (36). All sequences begin at the protein *N*-terminus and numbering is as for human CD8. Relationships to the Ig fold can be determined from References 29 and 37.


115nm?
LSGP
CARBOHYDRATES
N-LINKED
Fuc
Asn-GlcN-GlcN-Man
Man-GlcN-Gal-SA
Man-GlcN-Gal-SA
O-LINKED
Ser
Thr
-GalN-Gal-SA
SA
MHC
Class I
L-CA
Thy-1
10nm
Ganglio-
side
LIPID BILAYER
CYTOPLASM

molecules interact with class II and class I antigens, respectively, to control T cell responses (38). However, it now seems dubious as to whether this postulate is correct for CD4 antigen because antibody to this antigen blocks responses of T_{CD4} cells to an MHC antigen that has only the polymorphic domain of the β-chain of a class II MHC antigen spliced onto a class I heavy-chain structure (39). It is not obvious that CD4 should interact with the polymorphic domain of the class II MHC antigen, and steric considerations suggest that Tcr and CD4 could not interact with such a small segment at the same time. Antibodies to the Thy-1 antigen can trigger mouse T cell clones or hybridomas to release interleukin 2 and, with presenting cells, can be mitogenic for resting T cells (40). However, the physiologic relevance of this is also unclear because expression of Thy-1 antigen is not conserved on rat and human T cells (29).

For the antigens TL, Qa, and MRC OX-2 no functions nor effects of antibodies on cell responses have yet been identified.

Diversity in Carbohydrate Structures of the T Antigens

The carbohydrate structures of the surface antigens can be a prominent feature of their molecular architecture. This is illustrated in Figure 3 where a number of surface antigens are shown with their protein structures drawn roughly to scale. The area covered by a typical *N*-linked or *O*-linked carbohydrate structure is also shown and it can be seen that much of the surface of a molecule like Thy-1 would be covered by its three carbohydrate structures. Similarly carbohydrate is a major feature of L-CA but not of MHC class I antigens.

Diversity in carbohydrate structures can be seen between molecules on the same cell, and an extreme case is seen with the leukocyte sialoglycoprotein (LSGP), which is expressed on thymocytes and T lymphocytes but not on B lymphocytes (however, B blasts are LSGP-positive). LSGP

Figure 3. Some T lymphocyte glycoproteins drawn roughly to scale at a cell surface (adapted from Reference 19). The size of MHC class I is based on the dimensions of an antibody Fab fragment (37); Thy-1 on a single Ig domain (37); L-CA on the sequence of the molecule and the dimensions seen by electron microscopy after low-angle shadowing (41, 42); LSGP on the assumption that the polypeptide contains 340 amino acids (20) and that a polypeptide chain in extended conformation has a length of 0.34 nm per residue; the mode of integration of LSGP into the membrane is guesswork. The carbohydrates on the proteins are not shown to scale but the shapes shown above the molecules indicate tha cross-sectional areas for typical *N*-linked and *O*-linked carbohydrates (43). For L-CA the number of *N*-linked structures should approximate to the correct value, but the presence of *O*-linked structures, as indicated from the composition, is not shown.

has *O*-linked, but no *N*-linked, carbohydrate structures and the composition suggests that on average one in five amino acids is glycosylated (20). Thus this molecule is quite different from most other T cell antigens which seem to have more *N*-linked than *O*-linked carbohydrate. Mucins are a well-known category of molecules with a high content of *O*-linked structures and the mucin glycoprotein of submaxillary gland has been shown to have an extended linear structure (44). A similar structure for LSGP is suggested in Figure 3.

The carbohydrate structures attached to LSGP appear to have the core structure, Gal $\overset{\beta 1\text{-}3}{\rightarrow}$ Gal NAc $\rightarrow$ Ser/Thr, and this structure, but not the sialylated form shown in Figure 3, is recognized with high affinity by peanut lectin. LSGP from T cells does not bind to peanut lectin because it is fully sialylated although a subfraction of thymocyte LSGP does bind to the lectin (45). Thus a lack of sialic acid on LSGP of cortical but not medullary thymocytes is likely to be the major explanation for the preferential binding of this lectin to cortical thymocytes.

The function of LSGP is unknown but presumably this must involve the abundant carbohydrate structures. It has been suggested that the presence of this molecule on T but not B cells may be responsible for the more rapid transit time of T cells through lymphoid organs (20). This highly charged molecule may minimize interactions with other cells in the lymphoid organs. Humans suffering from Wiskott–Aldrich syndrome have leukocytes that appear to lack LSGP or to have a molecule with aberrant properties. Defects in LSGP may be responsible for this immunodeficiency disease (46).

Diversity in carbohydrate structures is also seen on other T cell antigens; for example, the L-CA molecules of thymocytes and T cells differ in their binding to lectins (45).

The exact roles of carbohydrates and the importance of their diversity remain a major puzzle.

Antigenic Heterogeneity in the Leukocyte-common Antigen

The L-CA is a major glycoprotein found on all lymphocytes and myeloid cells which can be calculated to cover about 15% of the lymphocyte surface (19). A model for L-CA based on visualization by electron microscopy after low-angle shadowing (41) is shown in Figure 3. The L-CA molecule has different apparent M_r forms on different lymphocytes with one band at M_r 180,000 being seen on thymocytes, four bands at 180,000, 190,000, 200,000, and 220,000 on T lymphocytes and one broad band around 240,000 on B cells (47). About 90% of the sequence of rat thymo-

cyte L-CA has been determined and from this it is concluded that about M_r 80,000 of the protein is inside the cell (42). The B cell form appears to derive from the same gene as the thymocyte form in the cytoplasmic part of the molecule but differences in the size of the mRNA for the different cell types suggest that there may be differences in sequence at the *N*-terminus (42). This remains to be substantiated.

Monoclonal antibodies that react with L-CA include those that bind to all forms of the L-CA glycoproteins whereas others can react with subsets of the molecules. One type of MAb, epitomized by the MRC OX-22 antibody that reacts with rat L-CA, serves as a T subset markers that splits the T_{CD4} subset into two fractions with different functions. The antibody labels all B cells and T_{CD8} cells as well as two-thirds of the T_{CD4} cells. On B cells the OX-22 antibody recognizes a major subfraction of the M_r 240,000 L-CA band while on T cells it reacts with a minor subset of the M_r 220,000, 200,000, and 190,000 but not the 180,000 band (47). A peptide that reacts with the Ox-22 antibody has been purified and partially sequenced (48). How this peptide is expressed in the full L-CA sequence remains to be resolved by further analysis at the cDNA level.

Table II. Size of Postulated Cytoplasmic Domains for Various Molecules Found at the Cell Surface of B or T Lymphocytes

Molecule	*Residues in cytoplasmic tail*	*Reference*
IgM or IgD	3	50
IgG	28	50
T receptor: α	5	13
β	5	
Class I MHC	31	34
Class II MHC: α	15	51
β	16	
CD3 antigen δ chain (p20)	44	14
Thy-1 antigen	Nil	52
CD4 antigen	38	33
CD8 antigen	29	23
MRC OX-2 antigen	19	21
L-CA	705	42

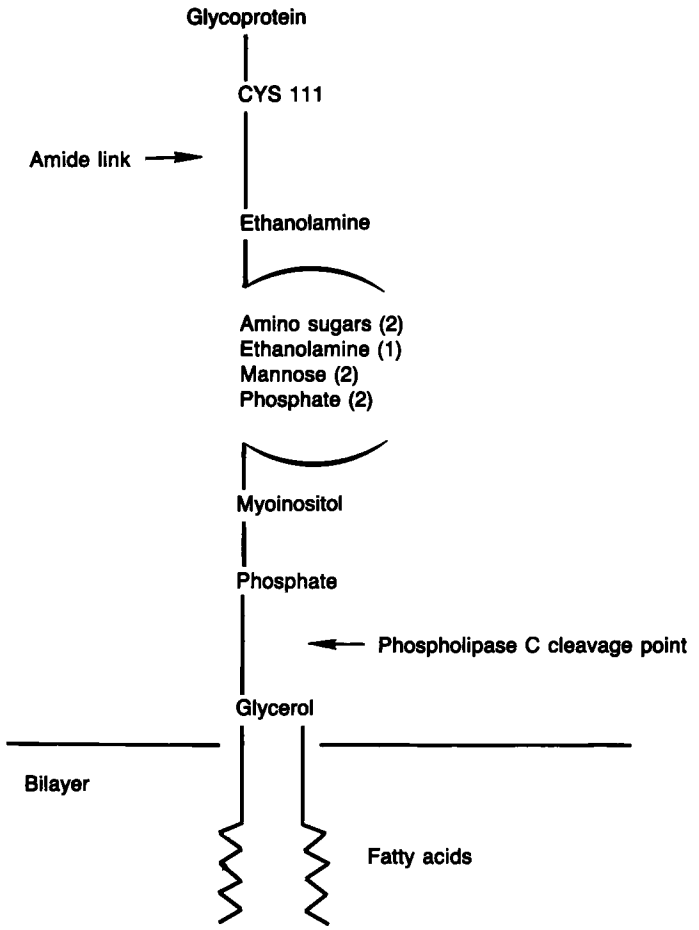


Figure 4. A working model for the structure of material at the *C*-terminus of the Thy-1 molecule. This is based on analysis of purified material that includes only the *C*-terminal cysteine residue plus attached nonprotein components (52, 54) and on the finding that a phospholipase C enzyme that is specific for phosphatidylinositol can release Thy-1 from the cell surface (55). Residues in addition to the components for which linkages are suggested are shown in the middle of the structure in amounts given in the brackets.

The heterogeneity of L-CA presents an unusual phenomenon, but the variations seen may well be of functional significance because the patterns seem to be strongly conserved between humans and rodents. Monoclonal antibodies specific for human L-CA, with similar specificities to MRC OX-22 have been described (49).

Attachment of Molecules to the Lipid Bilayer

One key function of cell surface molecules is to trigger intracellular processes after recognition at the surface. This might involve the opening of a channel for ion transport or direct interaction with the cytoskeleton. Studies on the T cell molecules suggest that, in all cases except Thy-1, the molecule is attached to the lipid bilayer by a single span of hydrophobic protein sequence. This is illustrated in Figures 2 and 3. In most cases the amount of sequence believed to be inside the membrane is small (Table II), and it seems unlikely that structures like the Tcr chains would interact directly with the cytoskeleton. One exception is the L-CA glycoprotein which has a very large cytoplasmic domain and is a strong candidate to mediate membrane–cytoskeleton interactions possibly involving fodrin (53). For other molecules transmembrane sequences may interact within the lipid bilayer.

Thy-1 antigen is a notable exception to the rule of integration via a transmembrane protein sequence. In this case there is a glycophospholipid structure covalently attached to the *C*-terminal cysteine 111 residue and this acts as the membrane-anchoring domain (52, 54, 55). A working model for this structure is shown in Figure 4. The nature of the Thy-1 tail has been controversial because from the cDNA sequence a protein sequence of 142 amino acids is predicted (56) but only 111 residues are found in the mature protein (23, 52). The extra 31 residues are believed to be cleaved off when the glycophospholipid tail is attached, and the situation for Thy-1 is similar to that seen for the major surface glycoproteins of trypanosomes (57). The Thy-1 data encapsulate the mysteries of how triggering occurs at the cell surface. This molecule does not cross the bilayer, yet antibodies to it can trigger lymphokine release and even mitogenesis.

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A Beginner's Guide to T Cell Receptor Genes

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Introduction

When asked to cite the year in which the study of immunology began a typical student of the 1970s would categorically answer 1974, the year in which Zinkernagel and Doherty (1) made the observation that antigen recognition by T lymphocytes is restricted by major histocompatibility complex (MHC) encoded gene products. A decade later the answer may have changed. A student of the 1980s might answer 1983, the year in which Hedrick, Davis, and their colleagues (2) and Mak and his colleagues (3) published the sequence of one of the chains of the T cell antigen receptor. This compression of immunologic history stands in marked contrast to the longevity of the Trudeau Institute, which no doubt will be thriving long after the immunological community has lost interest in T cell receptors.

Nevertheless, in 1985, antigen recognition by T lymphocytes is poorly understood in both molecular and in genetic terms. On the other hand, a wide array of experimental reagents (T cell lines and hybridomas of known specificity, antireceptor antibodies and cloned receptor genes) are now available and a detailed understanding of T cell antigen recognition may soon come to light. What I hope to accomplish in this chapter is to outline briefly the unusual aspects of T cell antigen recognition, to review the structure of the genes that encode these receptors, and to discuss an unusual configuration of receptor genes present in New Zealand White (NZW) mice.

T Cell Recognition of Antigens

Like B cells, T lymphocytes recognize antigens with a high degree of specificity and have memory for previous antigen exposure. In contrast to B cells, T lymphocytes are unable to recognize free antigens; these cells recognize antigens only on the surface of accessory or presenting cells (macrophages, dendritic cells, and even B cells) in the context of an MHC encoded gene product (1). The phrase "in the context of an

MHC encoded gene product" is deliberately vague and reflects the current understanding of these events. The weight of the evidence (4, 5) indicates that a single receptor (a disulfide-linked heterodimeric glycoprotein) mediates the recognition of antigens and MHC proteins but the precise molecular relationships between the involved parties (receptor chains, MHC proteins, and antigens) remains to be worked out. A molecular picture of T cell recognition is one of the goals held by most workers in the field. Restriction by a particular MHC haplotype is not encoded in the genome but is "learned" somatically, in the thymus, presumably as a result of selective processes occurring in this organ (6). The nature of this thymic selection resulting in the generation of MHC-restricted T cells is unclear. That the thymus influences the T cell repertoire has been clearly demonstrated (7), but a clear understanding of which specificities are positively selected and which are negatively selected has yet to be achieved.

T Cell Receptor Proteins and Genes

At this point, having stated what isn't known, one can more easily describe the current state of affairs in this field without fear of overstatement. The T cell antigen receptor is an 85,000-mol wt disulfide-linked heterodimeric glycoprotein, composed of an acidic α-chain and a more basic β-chain (8). This receptor was identified by raising antibodies against monoclonal T cell lines or hybridomas and finding that some of these antibodies inhibited antigen recognition by the immunizing T cell line but not by other T cell lines (9). In this sense these antibodies interfere with a T cell property that is clonotypic. Some of these clonotypic antibodies were converted into (B cell) hybridomas, and the resulting monoclonal antibodies were used to study the structure of these T cell surface proteins. Both isoelectric focusing and peptide mapping studies have demonstrated that these molecules display clonotypic variation, a property expected of antigen receptors (8).

The genetic basis of this clonotypic variation has come to light with the isolation of complementary DNA (cDNA) clones for the α- and β-chains of the receptor (2, 3, 10–12). The β-chain cDNAs were the first to be isolated (see above) and are located on chromosome 6 in the mouse (13) and chromosome 7 in man (14); their sequence reveals the presence of 300 base-pair (bp) variable (V), 12-bp diversity (D), 48-bp joining (J), and 519-bp constant (C) region gene segments (15). A functional β-chain gene is assembled from these gene segments by the joining of a D region segment to one of several J region segments. This is followed by the rearrangement of a V region segment to the previously formed DJ product

(16) (Figure 1). The β-chain gene segments are similar to those of the immunoglobulin-heavy (IgH) chain locus in that V, D, and J segments are involved in the assembly of a functional gene. The β-chain and IgH genes differ in that use of a D region is optional for the assembly of a β-chain gene but obligatory for the assembly of an IgH chain gene (17). The β-chain V regions are somewhat unusual compared to the V regions for other antigen receptor chains (IgH, Igk, Igλ, T cell receptor α). The germline repertoire of Ig and T cell receptor α-chain V region genes arose by series of gene duplications leading to the formation of V region gene families (18). The IgH V regions, for example, consist of six gene families, the largest having 40 and the smallest having six V region genes, respectively. The presence of V region families and the implied gene duplications are not an obvious feature of the β-locus. Most of the families

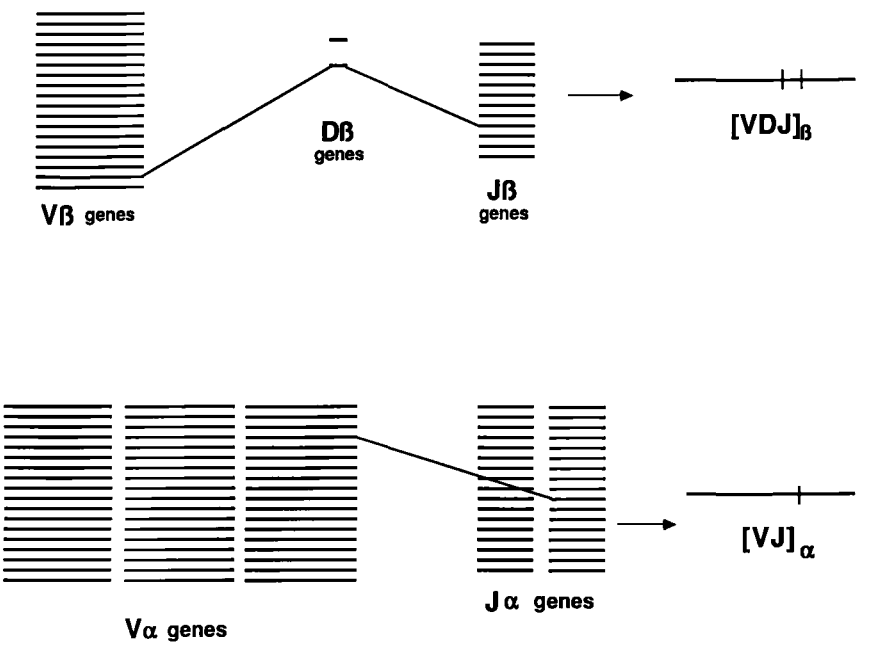

Figure 1. A diagrammatic representation of germline encoded T cell receptor gene segments. The β-chain genes are comprised of ≥20 Vβ genes, 2 Dβ genes, and 12 Jβ genes. A functional β-chain gene is formed by the rearrangement of a Dβ gene to a Jβ gene followed by the rearrangement of a Vβ gene to the previously formed DβJβ product. The α-chain genes are encoded by an unknown number of Vα gene segments and ≥25 Jα gene segments. There has not yet been a demonstration of the existence of Dα segments. A functional α-chain gene is formed by the rearrangement of a Vα gene to a Jα gene segment.

are extremely small (one member) although a few have two or three cross-hybridizing members (19). Furthermore, the total number Vβ genes is relatively small (20–30) (20). The low degree of homology between different β-chain V region genes indicates a lack of conservation in both the framework and hypervariable regions. Parenthetically, this property makes hypervariable and framework regions difficult to define because sequence comparisons between unrelated genes are difficult to interpret. One can surmise that the selective pressures at work during the evolution of the β-chain genes are subtly different from those operating on other V genes of the immune system.

The α-chain locus is encoded on chromosome 14 in mice and in humans and contains an unknown number of V region genes (21, 22); if there is any similarity to the Ig heavy and light chain loci, the α-chain locus may contain between 100 and 200 Vα genes. The Vα genes are similar to the Ig V genes in that they are organized into families (2–10 members/family) and presumably arose by gene duplication (23). Although there is as yet no evidence for the existence of D segments in the α locus (a property similar to Ig light chain genes), the arrangement of Jα segments is unlike that of any other locus encoding antigen receptor chains. There are at least 19 (and perhaps as many as 50) Jα gene segments. These segments are spread out over ≥63 kilobases (kb) of DNA upstream (5′) from the Cα exons (24). The reason for so many Jα gene segments isn't known but there is presumably an amplification of the size of the T cell repertoire owing to this feature of the the α-chain locus. A functional α-chain gene is assembled by the rearrangement of a Vα gene to one of the Jα gene segments (Figure 1).

The size of the germline encoded T cell repertoire can be estimated by multiplying two products:

The number (n) of different β-chains				The number of different α-chains		
(nVβ	× nDβ	× nJβ)	×	(nVα	× nJα)	
30	2	12		200	50	= ∼7.2 × 10^6.

As with any estimate, it has limitations. This number may be an underestimate because additional sequence diversity occurs at the sites of VβDβ, DβJβ, and VαJα recombination. On the other hand, this number may be an overestimate because it is not known whether all α- and β-chains can freely associate with one another. In the case of immunoglobulins a given heavy chain can associate with approximately 10% of light chains to form an antibody (25), and there may be a similar restraint on the number of α/β pairs which can form. There is a second

reason that this number may be an overestimate; for a given combination of Vα, Jα, Vβ, Dβ, Jβ gene segments encoding a particular T cell receptor, the replacement of one segment with one of its homologues (replacing Jα1 with Jα3, for example), may *not* lead to a *functionally different* receptor. There is evidence for this with immunoglobulins (26) and the extent to which this may be true for T cell receptors needs to be determined.

The γ-chain genes must be included in any discussion of T cell receptor chains. Isolated by Tonegawa and his colleagues, and mistakenly identified as encoding the α-chain, the γ-chain genes have many intriguing properties (27). The γ-chain locus, encoded on chromosome 13 in the mouse, contains V, J, and C region segments, and a functional gene is assembled by a V-J rearrangement (28). The locus is transcribed only in T cells and the peak of γ-chain transcription occurs early in thymocyte ontogeny (days 13, 14, and 15 of fetal development) (29). A low level of γ-chain transcription occurs in mature T cells restricted to class I MHC proteins. In spite of the data regarding γ-chain gene rearrangement and transcription, there is as yet no compelling evidence for the expression of a γ-chain protein or the requirement of γ-chain expression for the functioning of an MHC-restricted T cell receptor (30). Nevertheless, one has the sense that γ-chain genes might be important. There must be some selective pressure at work to preserve the T cell-specific rearrangement and expression of this locus.

T Cell Receptor Genes in New Zealand White Mice

Like any other locus, one expects that the α- and β-chain genes will be polymorphic. We have recently described an unusual allele of the β-chain locus occurring in New Zealand White (NZW) mice (31). There are normally two Cβ genes in genomes of mice, rabbits, and humans. Each Cβ gene is associated with a Dβ gene and a number (approximately six) of Jβ genes. The comparable spacing of Dβ, Jβ, and Cβ exons in each cluster indicates that this arrangement of gene segments arose by duplication (Figure 1). Furthermore, the sequence homology between the coding exons of Cβ1 and Cβ2 is remarkable. The nucleotide homology between the murine Cβ1 and Cβ2 coding exons is 96% (32) whereas in humans it is 97% (33). An examination of the sequence of the 3′ untranslated exons of Cβ1 and Cβ2 reveals a much lower degree of homology (46% homology in mice and 23% in humans). Assuming a rate of genetic drift of 1%/2.2 $\times$ 10^6 years, Gascoigne et al. (32) estimated that the duplication occurred over 120 $\times$ 10^6 years ago. Interestingly, there is no obvious functional difference between the two Cβ genes inasmuch as both Cβ1 and Cβ2 are expressed in both helper and cytotoxic T cells (34). Nevertheless, it

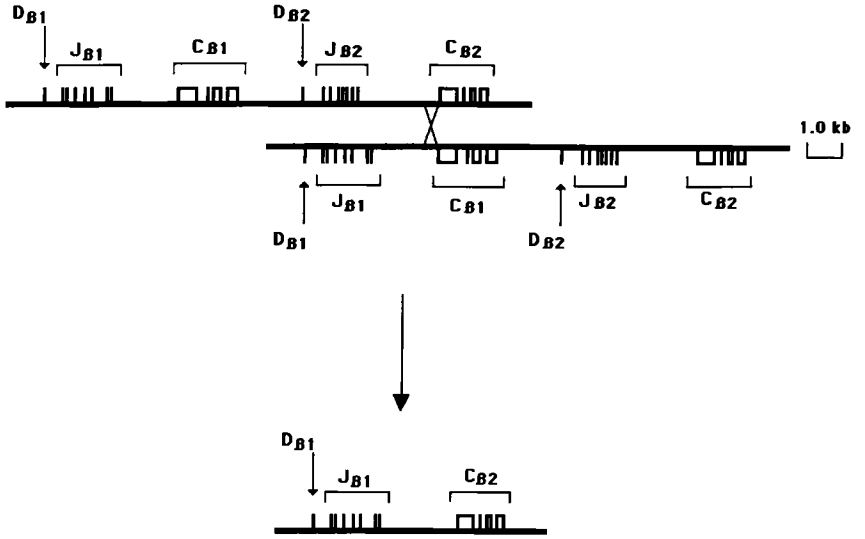

Figure 2. Probable recombination which generated the deletion in the NZW β-chain gene complex. A meiotic recombination between two chromosome 6 homologues may have produced a deletion of several β-chain gene segments, generating the allelic form of the β-chain locus, which is present in NZW mice.

is intriguing that this duplication has been maintained throughout 120 × 10^6 years of evolution.

While screening a number of inbred strains for restriction fragment length polymorphism, we identified an unusual allele of β-chain genes in NZW mice. An examination of NZW DNA with probes for the Cβ1, Cβ2, Dβ1, Dβ2, Jβ1, and Jβ2 gene segments demonstrated the occurrence of an 8.8-kb deletion of DNA containing the Cβ1, Dβ2, and Jβ2 gene segments. Thus, all T cell receptor β-chains in this strain are derived from a single set of Dβ, Jβ, and Cβ gene segments (Figure 2B). Restriction mapping allowed us to determine the approximate end points of the deletion, and we have postulated that the NZW allele was generated by an unequal meiotic crossover between two homologues of chromosome 6 (Figure 2).

The identification of a β-chain allele with one-half the usual number of Dβ and Jβ gene segments should allow us to determine the extent to which these gene segments contribute to the generation of a diverse T cell repertoire. To accomplish this, we are breeding BALB/c and C57BL6 mice that are congenic for the NZW β-chain genes. Eventually, we will have pairs of strains that differ only by the number of germline Dβ and

Jβ gene segments. We expect that mice lacking Dβ2 and Jβ2 genes may be unresponsive to certain antigens or may use alternate combinations of receptor gene segments in their response to these antigens. This follows from our working hypothesis that the selective pressure maintaining the duplication of β-chain gene segments during evolution is derived from the need for multiple Dβ and Jβ genes. Our long-range goal is to use these congenic strains to understand better the contribution of Dβ and Jβ gene segments in the diversification of the repertoire.

One characteristic of NZW mice makes the occurrence of this deletion somewhat intriguing. New Zealand White mice are not autoimmune, but when bred to New Zealand Black (NZB) or MRL mice, the resulting F1 hybrids develop autoantibodies and renal disease similar to that seen in human systemic lupus erythematosus (35). There is apparently something in the NZW genetic background that accelerates and worsens the slight autoimmunity seen in NZB and MRL mice. It is conceivable that the deletion of β-chain gene segments in the NZW genome contributes to the autoimmunity seen in [NZW/NZB] or [NZW/MRL] F1 animals. To test this formally, we have generated 185 [NZW/NZB] F1 × NZB backcross mice. We will attempt to correlate the presence of autoimmune disease with the presence of NZW β-chain allele. This experiment should give a definitive answer one way or the other. *If* the NZW β-chain allele is implicated in the generation of autoimmunity, then we will spend a great deal of effort determining the mechanism by which the NZW β-chain genes exert their effect.

Summary

Genes encoding the α- and β-chains of the T cell receptor have been cloned, and the genomic organization of these loci has been examined. Although there is an overall similarity in structure between T cell receptor genes and immunoglobulin genes, additional work is required to determine how the two unusual properties of T cells (restriction by MHC encoded proteins and somatic learning of MHC restriction in the thymus) are encoded in the structure of the receptor. Molecular probes for T cell receptor genes will also enable one to analyze the contribution of these loci to genetically determined diseases of the immune system.

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Regulation of T Cell Proliferation: Role of T Cell Antigen Receptors versus Interleukin 2 Receptors

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Introduction

More than 25 years have passed since the startling discovery by Nowell (1) that lymphocytes actually proliferate in response to mitogenic lectins. However, despite this pioneering breakthrough, the molecular mechanisms responsible for signaling T cell DNA replication and mitosis have only now become approachable, after painstaking biological and biochemical studies revealed the critical role of interleukin 2 (IL-2) in T cell proliferation (for review, see Smith [2]). For over 20 years it was believed that antigen/lectin activation set in motion a series of intracellular biochemical reactions that ultimately signaled DNA replication. Although attractive, this concept could not explain the requirement for the presence of non-T cells for the events to proceed, nor did it explain the long time interval, usually 2–3 days, that elapsed prior to the onset of detectable DNA synthesis. It is only now appreciated that T cell antigen-receptor activation is necessary for IL-2 production and IL-2 receptor expression, both of which comprise an extracellular polypeptide hormone-receptor mechanism that actually signals T cell DNA replication and subsequent mitosis.

In the past few years, several events have occurred that have permitted a crucial change in immunologic thinking, so that it could be realized that both T cell and B cell immunoregulation is directed by systems comprised of lymphokine polypeptide hormones and receptors. First, as regards T cell proliferation, the discovery by Morgan et al. (3) that T cells can be maintained in long-term proliferative culture using conditioned medium from lectin-stimulated mononuclear cells, suggested for

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the first time that antigen or lectin were not solely responsible for T cell growth, but that mononuclear cell products might also be important. Despite this finding, several years of detailed biologic and biochemical studies were necessary before it was discovered, and finally established, that the active moiety in conditioned media responsible for T cell growth is a single-peptide hormone, now termed IL-2. For these experimental advances, the establishment of a rapid, quantitative, unambiguous bioassay for IL-2 was essential (4, 5). Based upon an assay for erythropoietin that we had developed several years earlier (6), the IL-2 bioassay and biochemical studies established that the biologic activity is a single protein that only appears to be several molecules because of variable glycosylation (7). It proved critical to show that a single peptide was responsible for promoting T cell growth, because this greatly simplified the possible mechanisms responsible for the growth-promoting activity, and encouraged us to develop monoclonal antibodies reactive with IL-2 (8). In turn, IL-2 monoclonal antibodies were instrumental for the purification of large quantities of IL-2 to homogeneity so that the determination of the amino acid sequence could be accomplished (8). In addition, the monoclonal antibodies were necessary to prove unequivocally that IL-2 is responsible for T cell DNA replication and mitosis, rather than antigen or lectin: anti-IL-2 completely abrogates antigen/lectin-dependent T cell proliferation, and homogeneous IL-2 supplied in excess circumvents this inhibition (8). Thus, IL-2 could actually be defined as the molecule that functions to signal T cell proliferation.

The experience we gained in the biochemical isolation of IL-2 also led us to the purification of biosynthetically radiolabeled IL-2 and to the development of an assay for radiolabeled IL-2 binding (9). Therefore, for the first time, experiments clearly showed that the mechanism whereby IL-2 influences T cells occurs via specific membrane receptors. Consequently, these experiments not only established the precedent that lymphokines are similar to classic endocrine polypeptide hormones (10), but they also opened the way toward experiments to understand the biochemical and molecular cellular reactions triggered by IL-2.

Given this new awareness, we have spent the past several years focused on experimental approaches to understand T cell growth as it is regulated through the production of IL-2 and the expression of IL-2 receptors. In this regard, the dissection of the IL-2-receptor system is confounded somewhat by its very nature (11). Because T cells themselves both produce and respond to IL-2, it is imperative to separate activation steps that trigger the IL-2 receptor apparatus from those stimulated by IL-2 itself. When freshly isolated T cells are placed into culture in the pres-

ence of antigen or polyclonal activating agents, within a few hours IL-2 is secreted into the culture medium and IL-2 receptors begin to appear at the cell surface. Thus, it has been difficult to separate the events that occur upon activation of the T cell antigen-receptor complex from those that are triggered via IL-2 receptors. Moreover, compounding the complexity of the system, T cells respond asynchronously to antigen-receptor triggering; i.e., some cells become activated before others (12). Finally, of even greater importance, some cells are capable of proliferating in response to the IL-2 that they themselves produce in an autocrine fashion, whereas others utilize IL-2 produced by neighboring cells in a paracrine fashion (13). Consequently, one must isolate, characterize, and synchronize each aspect of the system of IL-2 hormone receptors before meaningful approaches to the molecular mechanisms responsible for T cell proliferation can be undertaken.

Mechanisms Regulating the Transient Nature of the T Cell Proliferative Response

One of the unique characteristics of the immune system is the absolute specificity of reactivity, especially when considered in regard to the vast diversity of potential responses. With respect to T cell proliferation, it is now recognized that the specificity of antigen reactivity is maintained via a strict antigen dependency on the expression of IL-2 receptors (9–11). In the absence of antigen, IL-2 receptors are undetectable and the majority of cells are unresponsive to IL-2. Moreover, the frequency of IL-2-responsive cells among circulating human peripheral lymphocytes is low and consistent with the IL-2 receptor assay results: only 1 in 500 circulating T cells proliferates in response to exogenous IL-2 in the absence of antigenic stimulation. By comparison, if the cells are exposed to polyclonal activating agents such as mitogenic lectins, phorbol esters, and antibodies reactive to T3 determinants, virtually all identifiable T cells express IL-2 receptors and become IL-2-responsive.

The biologic necessity of maintaining antigen specificity, and the observed dependency of the expression of IL-2 receptors on antigen receptors, indicate that both antigen and antigen receptors must persist for IL-2 responsiveness to continue. In vivo, during an effective host immune response, antigens are cleared progressively. Accordingly, as the concentration of antigen decreases, the signals emanating from the antigen-receptor complex that stimulate transcription of the IL-2 receptor gene are attenuated, thereby resulting ultimately in the disappearance of IL-2 receptors and IL-2 responsiveness. In addition, in vitro studies have shown that antigen receptors themselves disappear after the initial antigen-

receptor interaction and remain undetectable for several days, even if antigen is removed (14, 15). Therefore, two phenomena restrict IL-2-dependent T cell clonal expansion via their control over the expression of IL-2 receptors: the disappearance of antigen and the disappearance of antigen receptors, both of which guarantee the cessation of signals necessary to maintain expression of the IL-2 receptor gene.

The consequences of the dynamics of the expression of IL-2 receptors as they relate to antigen-receptor interaction are shown in Figure 1. Freshly isolated human peripheral blood mononuclear cells do not express detectable IL-2 receptors. If activated via anti-T3, which stimulates all cells expressing mature T cell antigen-receptor complexes (termed Ti-T3), the cells express IL-2 receptors asynchronously, so that a full 72 hours elapses before all of the cells within the population express detectable receptors. Moreover, this asynchrony is manifested by the heterogeneous number of IL-2 receptors expressed by the cells comprising the population. For example, there is a logarithmic-normal distribution of IL-2 receptors that varies 1,000-fold among the cells within the population as detected by cytofluorometric analysis at the peak of IL-2 receptor expression, (Figure 1, *inset*).

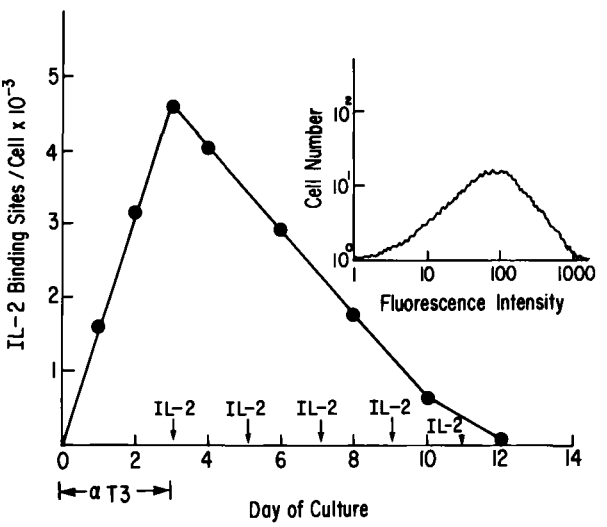

Figure 1. The appearance and disappearance of high affinity IL-2 receptors. Human peripheral mononuclear cells were cultured for 3 d in the presence of anti-T3 (Ortho Diagnostics, Raritan, NJ, 1:10,000). Thereafter the cell cultures were diluted 5-10 fold every 2 d, when immunoaffinity-purified IL-2 was replenished (125 pM). *Inset*: Cytofluorometric analysis of cells cultured for 3 d, as defined using anti-Tac.

When anti-T3 is removed from the culture at 72 hours and the cells are maintained in IL-2, the density of IL-2 receptors declines progressively over the next 10–12 days. Because expression of IL-2 receptors is dependent upon activation of the Ti-T3 complex, it is to be expected that the cell population will cease to express IL-2 receptors after the removal of anti-T3. However, a second mechanism, dependent upon IL-2, also guarantees the loss of biologically relevant IL-2 receptors: IL-2 binding to high-affinity IL-2 receptors actually promotes their rapid disappearance, while in their place low-affinity receptors accumulate gradually (16). The mechanisms responsible for this IL-2-directed switch in the kind of IL-2 receptors expressed have not been entirely delineated. However, an IL-2-mediated accelerated internalization of high-affinity IL-2 receptors is clearly operative: in the absence of IL-2, the membrane half-life of high-affinity IL-2 receptors is about 2.5 hours, whereas in the presence of IL-2, this time interval decreases to only 20 min (16, unpublished observation). Therefore, this 7.5-fold-accelerated internalization of high-affinity IL-2 receptors certainly contributes to the disappearance of this class of receptors. However, this mechanism can not account for the 10-fold increase in detectable low-affinity receptors that is mediated by IL-2, especially because we find no evidence for receptor recycling. An example of the reciprocal switch in IL-2 receptor expression promoted by IL-2 is shown in Figure 2, where G_0/G_1-synchronized, IL-2 receptor-positive cells were exposed to IL-2 or left untreated. There was a 25% diminution in detectable high-affinity receptors (6,500 vs. 4,500 sites/cell) after exposure to IL-2 for 14 hours, whereas the number of low-affinity receptors increased from undetectable levels to 40,000 sites/cell. Of particular relevance, the anti-Tac monoclonal antibody (17) recognizes an epitope common to both high-affinity and low-affinity receptors, and therefore does not discriminate between them. Moreover, using a complementary DNA (cDNA) obtained with the anti-Tac antibody and messenger RNA (mRNA) isolated from an IL-2 receptor-positive leukemic cell line (MT-2) (17), it can be seen that IL-2 itself induces the expression of Tac mRNA. Because the Tac cDNA encodes only low-affinity IL-2 receptors when transfected into fibroblasts (18), it appears that IL-2 interaction with high-affinity IL-2 receptors results in the expression of mRNA that encodes solely low-affinity IL-2 receptors. Yet to be realized is the effect of IL-2 on the genes encoding biologically relevant, high-affinity receptors, which must await their isolation and characterization.

Regardless of the exact nature of the mechanism(s) responsible for the IL-2-directed switch in the kind of IL-2 receptors expressed, the conclusion becomes inevitable that IL-2 ultimately controls the magnitude of

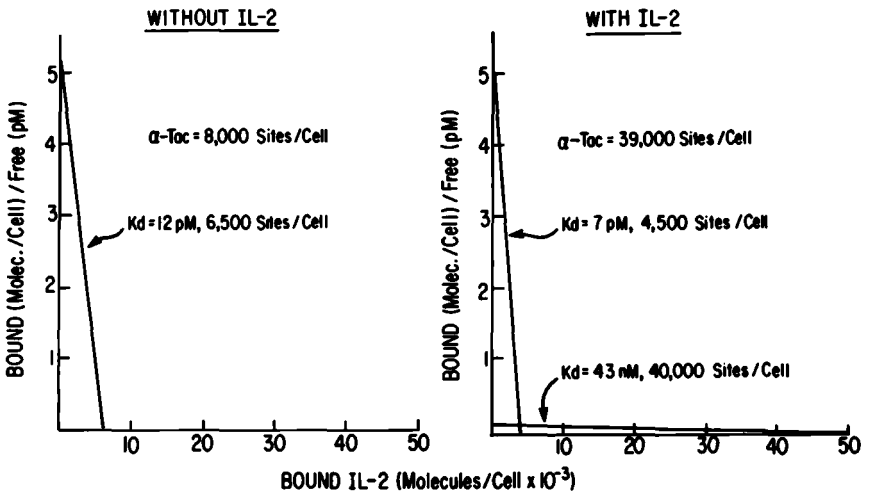

Figure 2. IL-2 induction of low affinity IL-2 binding sites. Human peripheral mononuclear cells were cultured for 12 d with IL-2, and for 2 d without IL-2. IL-2 receptor re-expression was achieved by a 6 h culture with phorbol dibutyrate (50 ng/ml). The cells were then exposed to immuno affinity-purified IL-2 (125 pM) for 14 h prior to the binding assays.

its response by regulating the expression of its own receptors. The biologic importance of this phenomenon relates to the probability that this mechanism occurs in other polypeptide growth factor systems, particularly those where autocrine growth regulation controls division of a particular cell type. In this regard, it is hardly coincidental that high-affinity and low-affinity receptors have been described to bind several other polypeptide hormones, including platelet-derived growth factor, epidermal growth factor, and nerve growth factor (16). Moreover, it is worthy of emphasis that studies have yet to be performed in other cell systems that mimic those we have used for T cells: the IL-2-T cell system provides the unique opportunity to study freshly isolated, normal tissues, in addition to established IL-2-dependent T cell lines and neoplastic IL-2-independent T cell lines. By comparison, fibroblast and epithelial cell systems provide only established cell lines for examination.

Conclusions

The discovery and establishment that antigen-dependent T cell proliferation is mediated by a polypeptide hormone-receptor mechanism composed of the lymphokine IL-2, for the first time, have opened immunology to areas common to cell biology, physiology, and endocrinology. Thus, we are now concerned with the molecular mechanisms of ligand-receptor

interaction, the mechanisms of transmembrane signaling, and hormone production and receptor expression in the regulation of the immune response. Consequently, it is especially intriguing that T cell proliferation is switched on and off by a reciprocal regulation of IL-2 receptor expression. For example, activation of the T cell antigen-receptor complex induces the expression of high-affinity IL-2 receptors, which are only expressed transiently. The disappearance of the high-affinity IL-2 receptors can now be attributed to several mechanisms. First, as antigen is cleared through the reticuloendothelial system, it is no longer available to trigger the antigen receptors. Secondly, antigen receptors themselves disappear after stimulation, thereby ensuring the cessation of antigen-receptor interaction. Finally, IL-2 itself promotes the disappearance of biologically relevant high-affinity receptors by accelerating their removal from the cell surface, and as well, by switching the cellular expression of the binding sites of IL-2 to those that are inactive and of low affinity. Of particular importance, this swtich appears to occur at the level of mRNA expression, and may well comprise a general mechanism whereby hormones control the magnitude of their own biologic responsiveness.

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T Cell Development and T Cell Tolerance

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Introduction

It is generally believed that T lymphocytes develop from immature hemopoietic cells in the thymus. What is unknown, however, is whether there is only functional maturation of T cells in the thymus, or whether, in addition, the generation of diversity of antigen-specific receptors occurs intrathymically. The thymus contains various subpopulations of lymphocytes but little is known about their precursor–product relationship. We have addressed some of these questions by analyzing, in organ culture in vitro, the development of the earliest hemopoietic cells that colonize the thymus as well as by studying the expression of receptors for antigen by various thymocyte subpopulations. The experiments indicate that the diversity of antigen recognition is generated intrathymically.

Because thymocytes are tolerant to many self antigens, one has to postulate that tolerance induction to antigens recognized by the α,β-heterodimeric T cell receptor must also be induced intrathymically. We have studied the cellular requirements for this process by transplantation of major histocompatibility (MHC)-different thymuses into nude mice. The experiments indicate that hematopoietic rather than epithelial cells are required for tolerization and that these cells include antigen-presenting cells.

Development of Thymocyte Subpopulations Defined by Surface Antigens

The murine thymus is colonized by hemopoietic cells at days 11–12 of gestation (1). At day 14 one finds a population of large, dividing cells, which are functionally incompetent and express Thy-1 and H-2 antigens but neither Ly-2 nor MT4 antigens (double-negative lymphocytes) (Table I). If the thymus is removed at this stage and put into organ culture, an exponential increase in cell numbers is observed during the next 6 days. During the same period one observes differentiation of lympho-

Table I. Surface Markers on Embryonic Thymocytes Differentiating In Vitro

Days in culture	*Surface marker* $Ly\text{-}2^-, MT4^-$	$Ly\text{-}2^+, MT4^+$	$Ly\text{-}2^+, MT4^-$	$Ly\text{-}2^-, MT4^+$
	% cells			
0	100	0	0	0
2	79	6	13	1
4	23	51	14	12
6	10	70	10	10

cytes, which is characterized (*a*) by the appearance of first large and then small cells expressing Ly-2 as well as MT4 antigens (double-positive lymphocytes), and (*b*) the appearance of functional $Ly\text{-}2^+$, $MT4^-$ as well as $Ly\text{-}2^-$, $MT4^+$ cells (single-positive lymphocytes) (Table I) (2). It is apparent from the data in Table I that on many cells Ly-2 is expressed before MT4. To verify this impression and to analyze the differentiation of "early" $Ly\text{-}2^+$, $MT4^-$ cells the following experiments were conducted: thymocytes from 17-day-old fetuses were stained with Ly-2 and MT4 antibodies after treatment with either Ly-2 and MT4 antibodies and complement or complement alone. Treatment with Ly-2 antibodies plus complement removed all $Ly\text{-}2^+$ as well as $MT4^+$ cells, whereas treatment with MT4 antibodies plus complement removed all $MT4^+$ but not all $Ly\ 2^+$ cells (Figure 1). If the "early" $Ly\text{-}2^+$, $MT4^-$ cells are cultured for 24 hours (survival 40%), all cells acquire the expression of MT4 (Figure 2). Thus the sequence of differentiation may go from double-negative cells directly to double-positive cells (3) or from double-negative cells to "early" $Ly\text{-}2^+$, $MT4^-$ cells and then to (double-positive) $Ly\text{-}2^+$, $MT4^+$ cells. The immediate precursors for the "late" $Ly\text{-}2^+$, $MT4^-$ as well as $Ly\text{-}2^-$, $MT4^+$ cells are, however, not known. Although it is excluded that all double-positive thymocytes differentiate into single-positive cells (the turnover of the former populations is much faster than that of the latter [4]), it is uncertain whether a small proportion of double-positive cells contains the immediate precursors of single-positive cells. This differentiation step has not been observed in suspension culture of dissociated thymocytes (4) but may nevertheless occur in situ. On the other hand, single-positive cells may

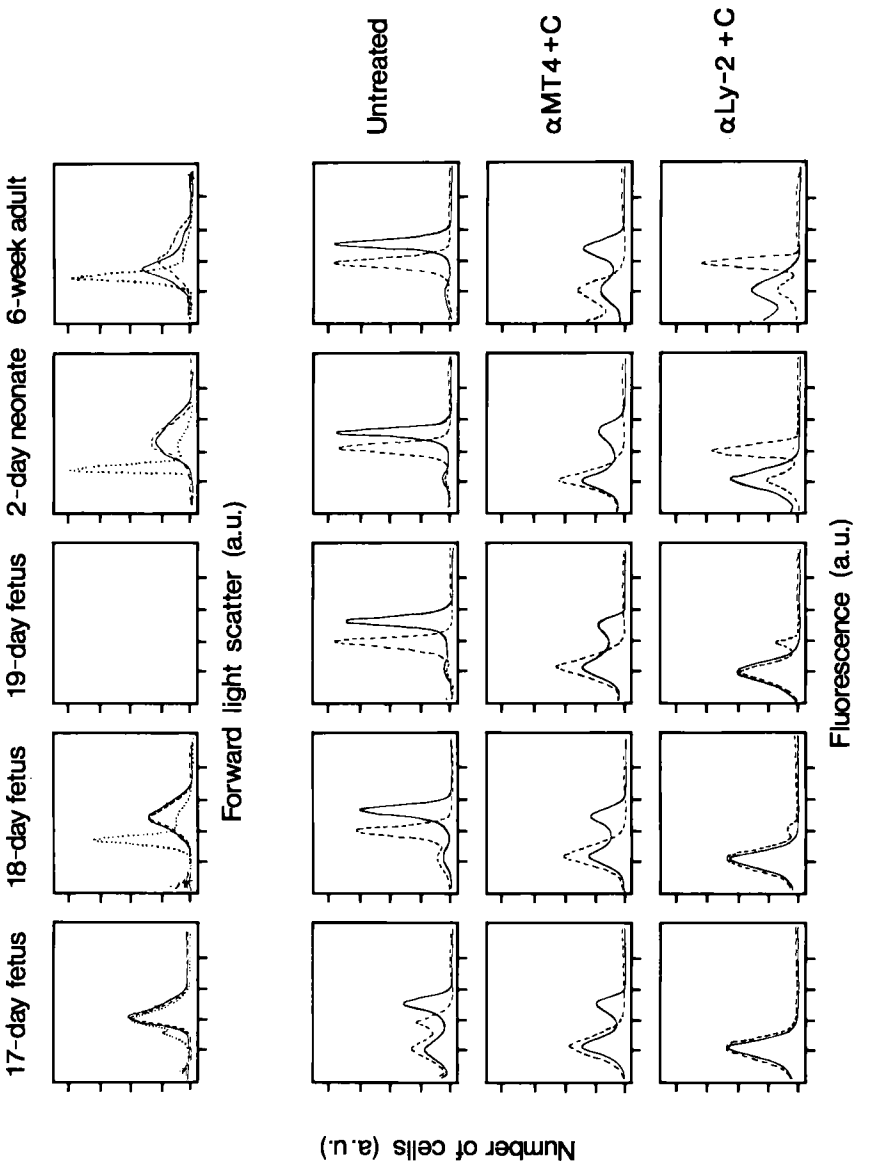

Figure 1. Staining of thymocytes from fetal mice of various ages with Ly-2 and MT4 antibodies before and after treatment with either MT4 or Ly-2 antibodies and complement (C). Solid lines indicate cells stained with Ly-2 antibodies; dashed lines indicate cells stained with MT4 antibodies; dotted lines (*top row*) indicate the size distribution of all thymocytes.

be directly derived from double-negative cells. It is also unknown whether the thymus contains a common precursor for both Ly-2$^+$, MT4$^-$ and Ly-2$^-$, MT4$^+$ cells.

Growth Requirements of Immature T Cells

A significant proportion (more than 50%) of the large dividing cells from 14-day-old embryos expresses interleukin 2 (IL-2) receptors as observed by staining and precipitation of receptors with IL-2 receptor antibodies (5–8). The proportion of thymocytes expressing IL-2 receptors diminishes with age and is confined to the dividing population of double-negative blasts (Table II) (6, 8). Even though not all dividing double-negative thymocytes express IL-2 receptors, the existence of these receptors on dividing cells raises the question whether or not the growth of some early

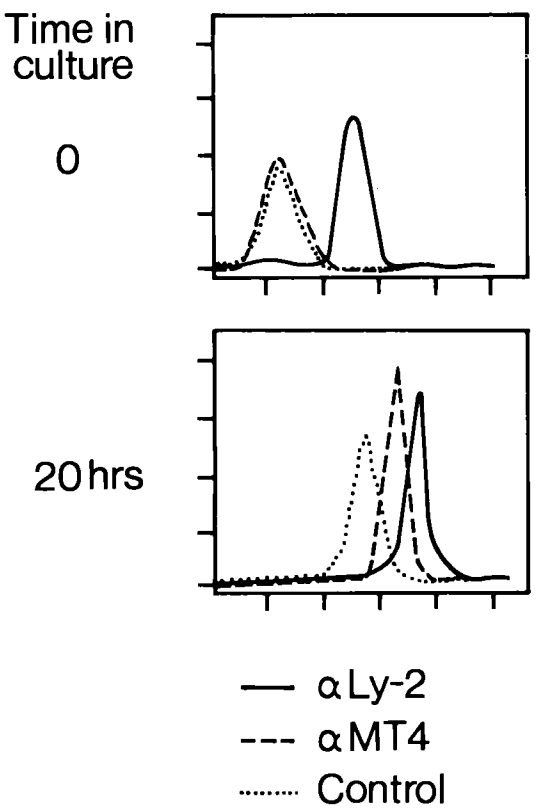

Figure 2. Thymocytes from a 17-day-old fetus treated with MT4 antibody and complement and stained with either MT4 or Ly-2 antibodies (*top*). Staining after 20 hours in culture (*bottom*) when 40% of the initially cultured cells were recovered.

Table II. Proportion of Interleukin 2 Receptor-positive Thymocytes in Embryonic Life

Embryonic age	*IL-2 receptor-positive*
days	*%*
14	90
15	72
16	20
20	4

Table III. Tritiated Thymidine Incorporation by Fetal Thymocytes and Activated T Cells (CTLL)

IL-2	*15-day-old embryonic thymocytes*	*CTLL*
	cpm	
–	6,700	9,200
+	6,900	115,400

T cells requires only the interaction of IL-2 with IL-2 receptors. In that case one would expect that IL-2 receptor-positive cells would continue to divide in the presence of IL-2 in vitro. This is clearly not the case (Table III) (8). In addition, it has been reported that most IL-2 receptors on double-negative thymocytes have low affinity for IL-2 (7). Finally, IL-2 cannot be induced by mitogens in fetal thymocytes expressing IL-2 receptors (2). Thus it is unlikely that the division of double-negative thymocytes depends solely on the binding of IL-2 to receptors on fetal thymocytes.

Various attempts were undertaken to grow IL-2 receptor expressing thymocytes in cultures containing IL-2. It has been claimed that addition of concanavalin A has some effect (6), but according to our experiment this effect is marginal (Figure 3). Other authors used phorbol myristate acetate (PMA), which can increase growth of fetal thymocytes considerably. The best growth is obtained by a combination of PMA and Ca^{++} ionophores. It was shown by blocking with anti-IL-2 receptor anti-

bodies that in this situation IL-2 was required for cell growth (9). After several days in culture, the dividing cells express Thy-1 but neither Ly-2 nor MT4 antigens. The cells can be cloned and their differentiation in vivo and in vitro is being studied.

T Cell Receptor Gene Expression

DNA Rearrangement

Gene rearrangement is a prerequisite for the expression of T cell receptors (10), as it is for the expression of immunoglobulin. T cell receptor gene rearrangements can be detected by using complementary DNA probes and restriction enzyme-digested DNA. Rearrangements of the β locus are easily detected on Southern blots (illustrated in Figure 4) using a probe hybridizing to the constant $C\beta_1$ and $C\beta_2$ gene segments. The same does not apply to the α-chain locus: the DNA containing the $J\alpha$ segments

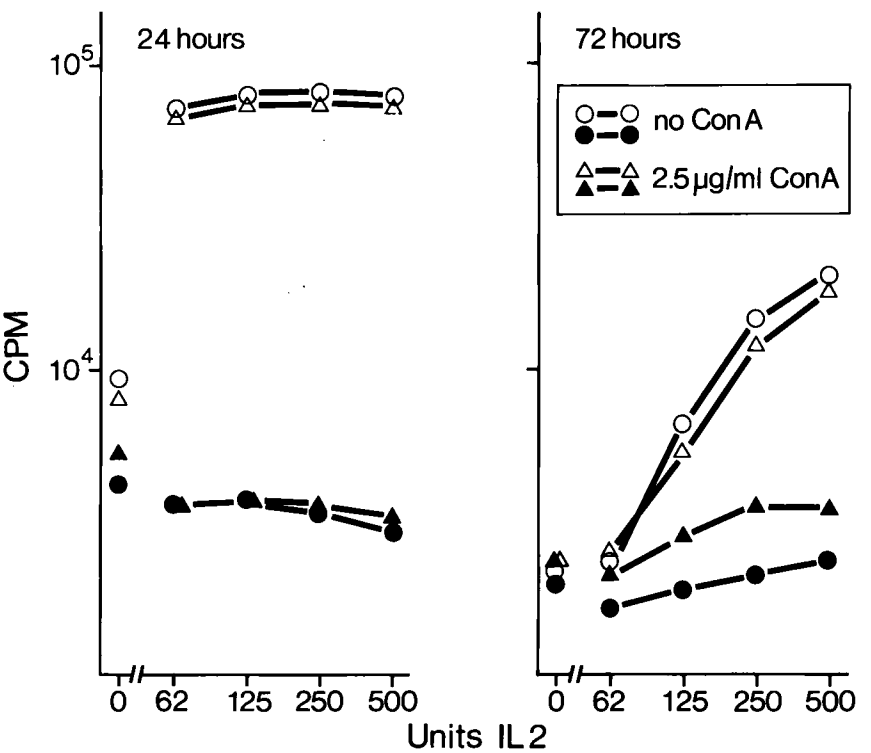

Figure 3. Thymidine incorporation by CTLL cells (*open symbols*) and thymocytes from a 14-day-old fetus (*solid symbols*) in IL-2-containing media in the presence or absence of concanavalin A (Con A) (2.5 µg/ml).

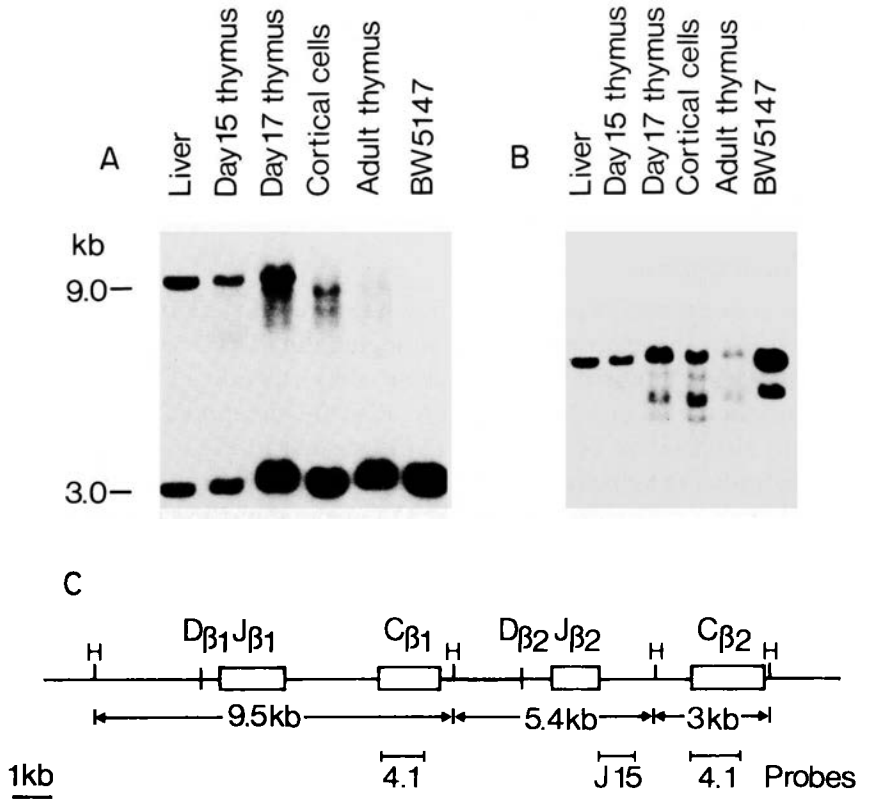

Figure 4. Southern blot analysis of the T cell receptor β-chain genes in cells from embryonic and adult mouse thymus. (A) DNAs digested with HindIII were separated on a 0.6% agarose gel and transferred to a Zeta probe membrane (BioRad Laboratories, Richmond, CA). Hybridization was with an insert of the 4.1 complementary DNA clone, containing approximately 70 base-pairs (bp) of a V-region gene segment, a J-region gene segment, and approximately 400 bp of either the $C\beta_1$ or the $C\beta_2$ gene of the T cell receptor β-chain. (B) After removal of the 4.1 probe, the same filter was hybridized with gel-purified insert of the J15 subclone. It contains the 900-bp ClaI-EcoRI fragment located approximately 90 bp downstream of the $J\beta_2$ gene locus. (C) Partial restriction map for the β-chain gene locus displaying the two D-region gene segments ($D\beta_1$ and $D\beta_2$), the two clusters of J-region gene segments ($J\beta_1$ and $J\beta_2$), and the two constant region gene segments ($C\beta_1$ and $C\beta_2$). The relevant HindIII sites (*H*) are indicated together with the lengths of the resulting fragments and the regions hybridizing to the probes 4.1 and J15. *Methods:* Fetal thymocytes were obtained from timed matings where the day of vaginal plug detection was designated as day 0. Cortical cells were isolated from adult thymocytes by pentose nucleic acid agglutination and anti-H-$2K^k$ plus complement. The resulting cells were more than 98% small Ly-2^+ $MT4^+$ thymocytes (data not shown). The T cell lymphoma, BW5147, was maintained in tissue culture. DNAs were isolated from cell suspensions or fresh tissue and digested with HindIII to completion. Probes were labled by nick-translation (4.1) or the oligo-labeling method (J15) to high specific activities (10^8 to 10^9

Table IV. Estimate of Beta Gene Rearrangement

Thymocyte subpopulations	*Proportion of cells with β-gene rearrangement*
	%
Day 15 fetal thymocytes	<30
Day 17 fetal thymocytes	75
Adult thymocytes	95
Ly-2$^+$, MT4$^+$ thymocytes	85

spans 63 kilobases (kb) so that the joining of V segments to some J segments is not detected by probes hybridizing to the Cα segment because of additional restriction enzyme sites between Cα and Jα. For this reason, only β-gene rearrangements have been analyzed in thymocyte subpopulations (11). In 15-day-old fetal thymocytes, there is relatively little rearrangement, whereas it is difficult to identify the β-germline bands in either double-positive thymocytes or in thymocytes from adult mice. Table IV shows a rough estimate, through using densiometric analysis, of the extent of rearrangement in various cell populations. By use of appropriate restriction enzymes one can show (10) that rearrangements have occurred to J segments in front of both Cβ_1 and Cβ_2 gene segments. The experiments do not indicate whether the rearrangements represent V-D-J or D-J segment joinings only. This question was analyzed by Northern blot analysis (Figure 5) probing for T cell receptor-specific RNA.

T Cell Receptor RNA and T Cell Receptor Protein

Complementary DNA probes hybridizing to α, β, and γ RNA (the γ locus shows many structural and sequence characteristics in common with the

cpm/μg). Hybridization to about 5 μg of DNA per lane was in 50% formamide, 1 M NaCl, 10% dextran sulfate, 0.2% bovine serum albumin, 0.2% Ficoll, 0.2% polyvinylpyrolidone, 50 mM Tris-HCl (pH 7.4), 0.1% $Na_4P_2O_7$, 1% sodium dodecyl sulfate (SDS), 150 μg/ml of sheared and denatured salmon sperm DNA with 3 × 10^6 cpm/ml of hybridization solution for 16 hours at 42°C. After hybridization filters were washed at 68°C twice in 3 × standard sodium citrate (SSC), 0.1% SDS, and twice in 0.3 × SSC, 0.1% SDS. Each wash was for 15 minutes. Exposure was overnight with two intensifying screens (Ilford Fast Tungstate, Ilford Ltd, Ilford, Essex, England). To remove the probe, the filter was incubated twice for 15 minutes at 90°C in 0.1 × SSC, 0.1% SDS. Sizes of restriction fragments hybridizing to the probes were measured using phage and DNA restriction fragments of known length run in parallel on the same gel.

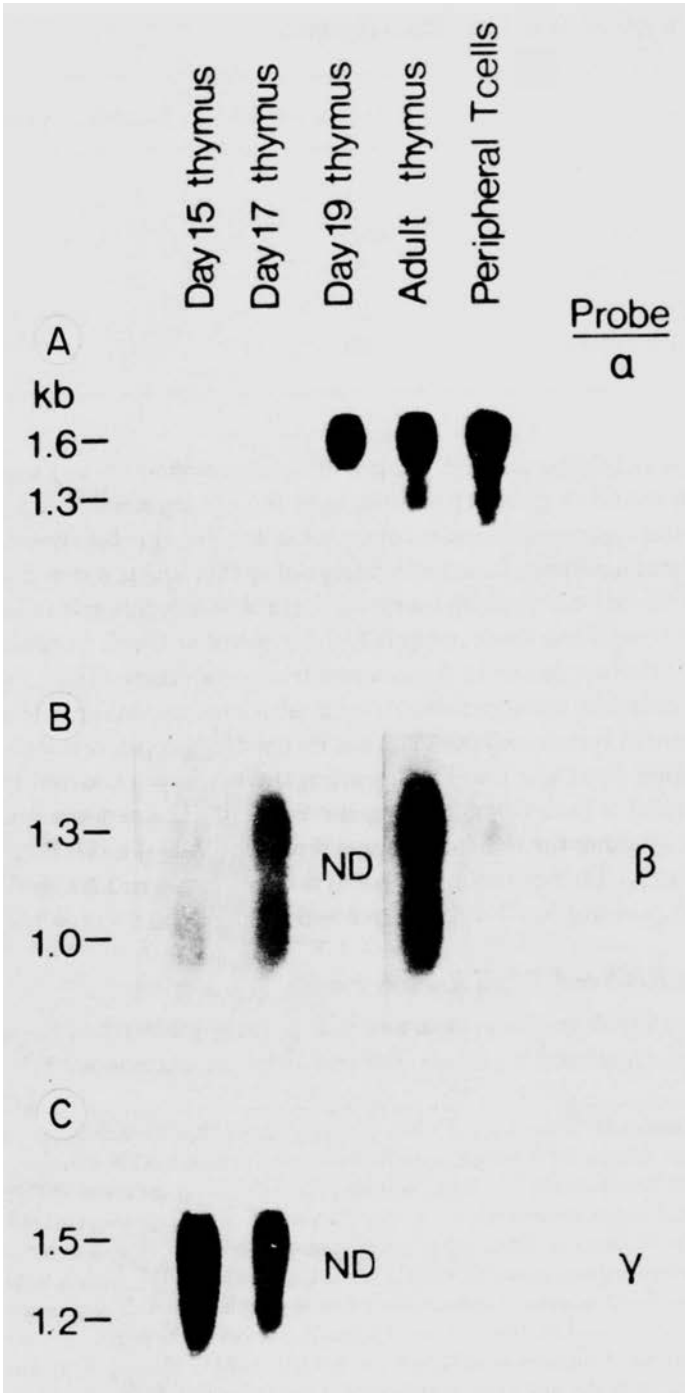
Day 15 thymus
Day 17 thymus
Day 19 thymus
Adult thymus
Peripheral Tcells
Probe
α
A
kb
1.6–
1.3–
B
1.3–
1.0–
ND
β
C
1.5–
1.2–
ND
γ

Table V. Alpha, Beta, Gamma Transcripts of Fetal Thymocytes

Days of gestation	*α RNA*	*β RNA*	*γ RNA*
15	−	−	+ +
17	(+)−	+	+ +
19	+	+	ND
Adult	+ +	+ +	−(+)

ND, not determined.

α and β locus) detect larger (1.6, 1.3, and 1.5 kb for α, β, and γ respectively) and smaller (1.3, 1.0, and 1.2 kb for α, β, and γ respectively) transcripts in thymocytes. It has been shown for the β gene that the larger transcript represents a full-length RNA whereas the smaller RNA represents a transcript from a DJ joining only. If only the larger transcripts are taken into consideration, then 15-day-old fetal thymocytes express γ, but not α or β, transcripts, 17-day-old fetal thymocytes express γ and β, but few α, transcripts, and adult thymocytes express α and β, but few γ, transcripts (Table V) (12, 13). It is clear, therefore, that double-negative cells that enter the thymus and contain precursors for functional

Figure 5. Northern blot analysis of T cells at various stages of development. Total RNA (20 μg) was separated on 1.2% glyoxal gels, transferred to Gene Screen (New England Nuclear, Boston, MA) and hybridized with an α-chain probe (A), or γ-chain probe (C). (B) ND, not done. *Methods:* B10.BR fetal thymocytes were obtained from timed matings where the day of vaginal plug detection was designated as day 0. RNA was isolated by the guanidinium isothiocyanate and CsCl gradient centrifugation procedures. RNA blots were hybridized in 1 M NaCl, 50 mM Tris, pH 7.5, 1% SDS, 0.2% polyvinylpyrolidone, 0.2% bovine serum albumin, 0.2% Ficoll, 100 μg/ml of denatured salmon sperm DNA, 10% dextran sulfate, and 1.5 × 10^6 cpm/ml oligo-labeled[30] probe (10^9 cpm/μg) at 68°C overnight. Filters were washed in 0.3 M NaCl, 60 mM Tris, pH 8, 2 mM EDTA, twice at 25°C for 10 minutes and twice at 68°C for 30 minutes, and twice in 10 mM NaCl, 6 mM Tris, pH 8, 2 mM EDTA at 25°C. Exposure was overnight with intensifying screens (Ilford Fast Tungstate). Sizes of RNA molecules were estimated from denatured phage DNA restriction fragments of known lengths run in parallel on the same gel. All probes are gel purified inserts. The α-chain probe is the 300-bp NcoI-AvaII fragment from the constant portion of the α-chain complementary DNA clone T1.2. The γ-chain probe represents the 1.3-kb insert of the tγ5 complementary DNA clone containing an almost full-length γ-chain complementary DNA as determined by comparison of this clone to published γ-chain complementary DNA clones.

thymocytes do not express α,β-heterodimeric receptors. Whether or not fetal thymocytes in the absence of an α transcript express a β,γ heterodimer is unknown, but it has been speculated that such a molecule may play a role in the selection of the T cell receptor repertoire (12, 13). The results obtained with probes for α and β transcripts together with those on the differentiation of 14-day-old double-negative fetal thymocytes in organ culture (2) indicate that precursors of T cells can enter the thymus prior to productive rearrangement of β- as well as α-gene segments and expression of full-length transcripts. Thus there is little doubt that the thymus is one organ where T cell diversity is generated. This does not exclude extrathymic differentiation of T cells but makes the proposition unlikely that T cell tolerance is acquired prethymically (14).

The question whether or not the nonfunctional double-positive thymocytes express full-length transcripts of the α and β locus has been addressed using purified double-positive thymocytes as well as double-positive thymomas. Double-positive thymocytes were prepared by agglutination with peanut agglutinin and by killing of cells expressing high amounts of class I MHC antigens. Of these cells 98.5% express both Ly-2 and MT4 antigens, and if RNA is isolated from this population, one obtains only one-sixth of that obtained from the same number of unfractionated thymocytes. By Northern blot analysis full-length α and β transcripts are easily detected (13). It can, however, not be excluded that these transcripts are derived from a few contaminating cells expressing high levels of RNA. This possibility has become more unlikely after recent experiments with Ly-2, MT4 double-positive, MEL 14-negative thymomas. Two out of two analyzed thymomas expressed high levels of full-length α and β transcripts. In addition, T cell receptorlike proteins are found on double-positive T cells as analyzed by gel electrophoresis (11) or by serologic methods (14). Both methods, however, fail to indicate whether all or only a proportion of double-positive thymocates express receptors for antigen.

Cellular Requirements for the Acquisition of Tolerance by Thymocytes

Experiments with parent into F_1 hybrid bone marrow chimeras have indicated that thymocytes differentiating in histocompatible thymuses acquire tolerance to MHC antigens (15). Subsequent results obtained with T cells from nude mice grafted with allogeneic thymuses showed that peripheral T cells from such mice were not always tolerant to graft-type MHC antigens (16). Because thymuses of different ages and different doses of irradiation were employed in the various experiments, we studied whether

Table VI. Thymidine Incorporation by Thymocytes Stimulated with Various X-irradiated Spleen Cells

Thymocytes 5×10^5	*Stimulators*		
	BALB/c	*B6*	*CBA/J*
	cpm		
B6-grafted BALB/c nu/nu 1[a]	500	3,600	7,900
B6-grafted BALB/c nu/nu 2[a]	1,400	6,900	7,400
BALB/c	300	10,500	15,500
B6	6,100	1,000	6,300

BALB/c nu/nu mice grafted with B6 deoxyguanosine-treated thymus.

the cellular composition of the thymus was critical for the induction of tolerance in thymocytes as well as peripheral T cells. We transplanted thymuses devoid of most hematopoietic cells after treatment with 2-deoxyguanosine (17) or untreated thymuses in allogeneic nude mice and monitored T cell tolerance in mixed lymphocyte culture by measuring proliferation as well as generation of cytolytic T lymphocytes (CTL). We found that thymocytes as well as peripheral T cells from mice grafted with deoxyguanosine-treated thymuses proliferated in mixed lymphocyte reaction and generated CTL when stimulated by spleen cells expressing graft-type MHC antigens (18) (Table VI, Figure 6). In contrast, thymocytes from mice grafted with untreated thymuses were unresponsive to graft-type antigens, but responded to third-party MHC antigens (Figure 6). The results indicate that (*a*) MHC antigens on thymus epithelium do not tolerize thymocytes and (*b*) MHC antigens of thymus epithelium cannot be presented in tolerogenic form by hematopoietic cells. The latter observation may be explained in two different ways: either hematopoietic cells cannot present shedded MHC antigens in the same configuration as present on epithelial cells expressing these MHC antigens, or antigen-presenting cells in the thymus cannot induce tolerance. To discriminate between the two possibilities, we studied whether, in mice grafted with deoxyguanosine-treated thymuses, thymocytes were tolerant to donor-type minor histocompatibility (4) antigens in the context of recipient-type MHC antigens. It is known that in vivo hematopoietic cells can

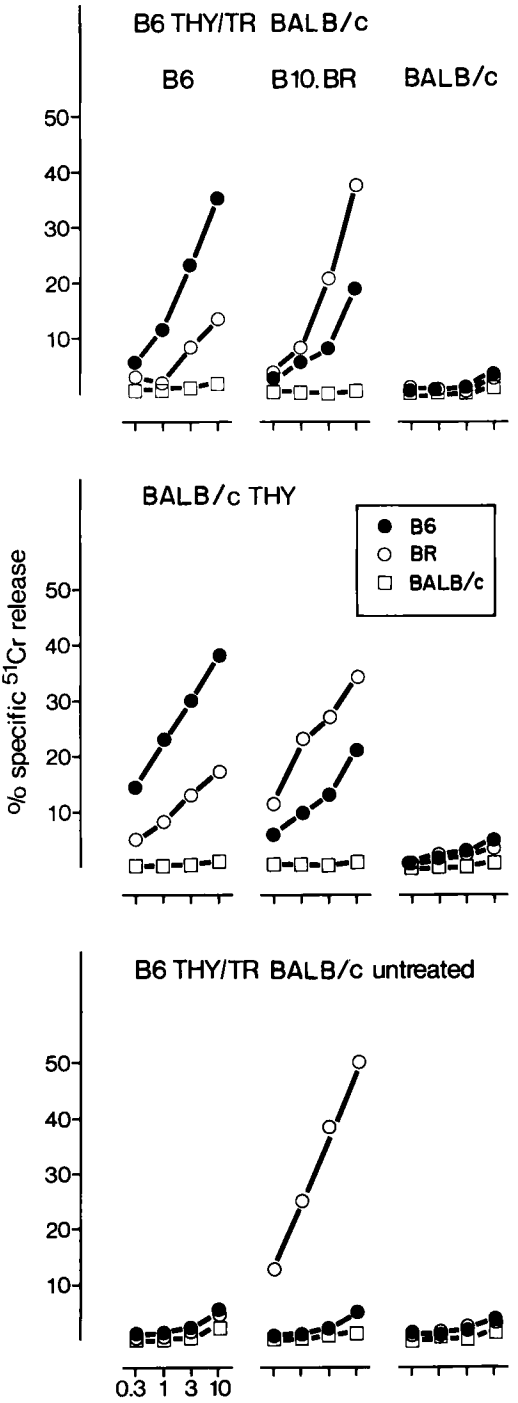

Figure 6. Lytic activity of CTL induced by B6, B10.BR (BR), and BALB/c stimulator cells on B6, B10.BR, and BALB/c targets. B6 THY/TR BALB/c (*top panel*) indicates B6 thymus, deoxyguanosine-treated, and transplanted into BALB/c nu/nu mice. B6 THY/TR BALB/c untreated (*bottom panel*) indicates untreated B6 thymus transplanted into BALB/c nu/nu mice. BALB/c THY (*middle panel*) indicates BALB/c thymus taken from a 6-week-old BALB/c mouse.

present minor histocompatibility antigens derived from other cells in such a way that, for instance, minor histocompatibility antigens from B6 (H-2^b) cells are presented by BALB/c (H-2^d) hematopoietic cells so that they induce H-2^d-restricted, B6 minor histocompatibility antigen-specific CTL, which lyse B10.D2 targets but neither B6 nor BALB/c targets (19, 20). If the same were true for tolerance induction, one might expect in a BALB/c nude mouse grafted with a deoxyguanosine-treated B6 thymus that B6 minor histocompatibility antigens derived from the thymus epithelium are presented by BALB/c hematopoietic cells colonizing the thymus and induce H-2^d-restricted tolerance. Thus T cells should be unable to generate B10.D2-specific CTL. On the other hand, T cells from such mice should be able to respond to DBA/2 cells presenting third-party minor histocompatibility antigens in the context of H-2^d MHC antigens. (One would expect to see this population of cells because, in response to minor histocompatibility antigens, CTL that are restricted

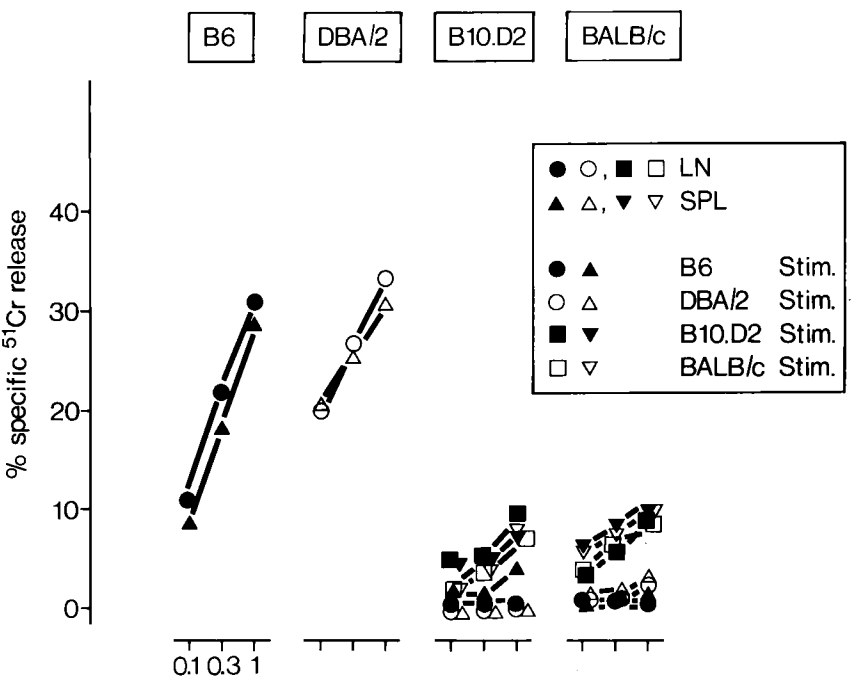

Figure 7. Lytic activity of CTL from lymph node (*LN*) or spleen (*SPL*) induced by B6, DBA/2, B10.D2, and BALB/c stimulators and tested on various target cells. All responding cells were taken from a BALB/c nu/nu mouse, immunized with anti-Thy-1-treated B10.D2 and DBA/2 spleen cells 8 weeks after transplantation. Cells were stimulated in vitro 14 days after priming.

by MHC antigens not expressed by thymic epithelial cells are regularly found [20, 21].) The predicted result was indeed observed (Figure 7): T cells from BALB/c nude mice grafted with deoxyguanosine-treated B6 thymuses generated CTL when stimulated by B6 or DBA/2 cells, but not when stimulated by B10.D2 cells. The experiments therefore indicate that hematopoietic antigen-presenting cells can tolerize thymocytes. Minor but not major histocompatibility antigens can be presented by such cells. The reason for this difference is unknown, but it may be that shedded MHC antigens cannot be presented in the same configuration as when they are expressed by epithelial cells. The results obtained with regard to tolerance are analogous to those that are observed in the activation of T cells: minor histocompatibility antigens are immunogenic when presented by hematopoietic cells that do not express these antigens (18), whereas the same is not true for MHC antigens.

In the afore-described experiments the lack of tolerance to MHC antigens expressed by grafted thymus epithelium was assessed by in vitro assays. Because the T cells contained CTL precursors and proliferating T cell precursors, one might expect that the thymus would be rejected if the animals were injected with homogeneous cells expressing donor-type MHC antigens. We have conducted such experiments 8 weeks after transplantation and we have failed to detect rejection. Whether this failure reflects in vivo tolerance not manifest in in vitro assays, or a failure of activated T cells to reach the graft, requires further investigation.

Conclusion

The Ly-2, MT4 double-negative lymphoblasts present in 14-day-old fetal thymus contain the precursors of nonfunctional Ly-2, MT4 double-positive as well as of functional, single-positive $Ly\text{-}2^+$, $MT4^-$, and $Ly\text{-}2^-$, $MT4^+$ thymocytes. The double-negative precursors can be grown and cloned in IL-2-containing media in the presence of PMA and Ca^{++} ionophores. Although further differentiation of these cells is not observed in suspension culture in vitro, they do differentiate in organ culture in vitro. Factors responsible for differentiation are being analyzed.

Double-negative cells enter the thymus prior to α- and β-gene rearrangement and expression of full-length α and β transcripts. The results leave little doubt that the thymus is one organ where T cell diversity is generated. Because diversity is generated intrathymically and thymocytes acquire functional maturity, it follows that prethymic tolerance (if it exists) cannot be a sufficient explanation for tolerance of thymocytes, i.e., some tolerance must be acquired intrathymically.

The cell that can induce intrathymic tolerance to major and minor histocompatibility antigens is hematopoietic rather than epithelial, and includes cells that can present minor but not major histocompatibility antigens derived from thymic epithelial cells.

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Functional Heterogeneity of Inducer/Helper T Cells — Lymphocytes That Produce Interleukin 2 Are Phenotypically Distinct from Those That Provide Help for B Cells

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Introduction

Earlier work with alloantibodies in the mouse has revealed a phenotypic heterogeneity of thymus-derived lymphocytes (T cells) and has shown that this heterogeneity correlates with a functional one (1). Subsequent studies using monoclonal antibodies performed in rats, humans, and mice have confirmed and extended these earlier findings (2–4) and have demonstrated that T cells can be assigned to two main groups—namely inducer/helper T cells that express the CD4 antigen (5), and cytotoxic/suppressor cells with CD8 molecules on their surface (5).

Inducer/helper T cells have been shown to play essential roles in both humoral and cell-mediated immunity in that they are required for the differentiation of B cells to antibody-secreting cells and of cytotoxic T cell precursors to mature effector cells (1–4, 6, 7). This diversity of function raises the question of whether inducer/helper T cells are themselves functionally heterogeneous with different subpopulations involved in these two arms of the immune response. This review describes data, obtained from studies in the rat, that demonstrate that inducer/helper T cells are indeed heterogeneous with respect to function. This work has been made

Acknowledgments

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possible by the production of a monoclonal antibody, MRC OX-22, that labels only a subset of inducer/helper T cells in this species.

Production of MRC OX-22 Monoclonal Antibody and the Tissue Distribution of the MRC OX-22 Antigen

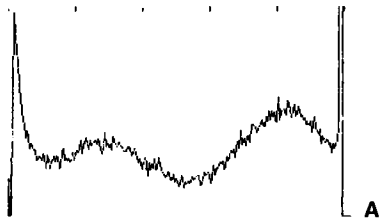
MRC OX-22 monoclonal antibody was derived by a fusion of spleen cells from a Balb/C mouse immunized with phytohemagglutinin-activated rat lymph node cells with NS1 myeloma cells (8). Cloned hybridomas were used to provide antibody-containing tissue culture supernatant and were also injected into mice to obtain high-titer ascitic fluid.

The tissue distribution of the antigen recognized by the antibody was determined by flow cytofluorography of single-cell suspensions, by immunoperoxidase staining of cryostat sections of solid organs, and by quantitative absorption by tissue homogenates (8). For the flow cytofluorographic analysis, a range of monoclonal antibodies were used, both singly and mixed with MRC OX-22 antibody to determine which cell type was reacting with this monoclonal reagent. The tissue distribution data are presented in Table I. The fluorescence histogram, obtained on flow

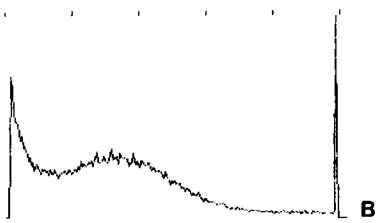
Table I. Tissue Distribution of the MRC OX-22 Antigen

Tissue[a]	*Cells reactive with MRC OX-22 antibody*
TDL, splenocytes	All B cells All T cells of cytotoxic/suppressor phenotype (i.e., $CD8^+$ cells) 65–70% of $CD4^+$ cells
Thymus	1–2% of all thymocytes. Positive cells scattered through cortex and concentrated at the corticomedullary junction
Bone marrow	46% of bone marrow cells including those of B cell lineage
Erythrocytes, kidney, liver, brain, skin	All negative

a. Flow cytofluorographic analysis was carried out on TDL, splenocytes, thymocytes, and bone marrow cells. Cryostat sections were prepared from spleen, thymus, liver, brain, and kidney and quantitative absorption analysis carried out on erythrocytes, splenocyte, thymocytes, and homogenates of kidney and liver.



ox-22 label - undepleted TDL



ox-22 label - TDL depleted of
B cells and T cytotoxic cells

Figure 1. MRC OX-22 monoclonal antibody (MAb) labels a subset of $CD4^+$ cells from rat TDL. One fraction of rat TDL was incubated with MRC OX-22 monoclonal antibody, washed, and incubated with fluorescein-conjugated rabbit $F(ab')_2$-anti-mouse Ig (RAM-FITC). The other fraction was first depleted of B cells and $CD8^+$ T cells by a rosetting technique (9) and the residual cells were then labeled with MRC OX-22 antibody followed by RAM-FITC. Fluorescence histograms were generated on a Becton-Dickinson FACS II machine. (A) Undepleted TDL. (B) TDL depleted of B cells and $CD8^+$ cells.

cytofluorographic analysis of rat thoracic duct lymphocytes (TDL) labeled with MRC OX-22, is shown in Figure 1 A. After removal of B cells and $CD8^+$ T cells from TDL and labeling the residual cells with MRC OX-22, a bimodal fluorescence profile was obtained (Figure 1 B) that clearly represented the negative and weakly positive peaks of the trimodal profile observed with unfractionated TDL. The cells remaining after the removal of the B cells and $CD8^+$ cells were shown to be 97-98% $CD4^+$ TDL. Detailed analyses indicated that B cells, which made up ~45% of the TDL, were all MRC OX-22 positive as were the $CD8^+$ cells (7-8% of TDL). In contrast, the $CD4^+$ cells, which made up all but a very small

percentage of the remaining cells, were heterogeneous with about two-thirds MRC OX-22 positive and one-third MRC OX-22 negative (8).

Biochemical Characterization of the MRC OX-22 Antigen

Preliminary experiments indicated that the MRC OX-22 antigen had a molecular weight of ~200,000, i.e., within that of the higher molecular weight band of the leukocyte-common antigen (L-CA). To investigate the possible relationship between these two molecules, the sialic acid residues of glycoproteins of TDL, and of B cells and T cells purified therefrom, were labeled by the [^{3}H]Na borohydride method (10) and solubilized in detergent. The extract so obtained was passed through MRC OX-22 or MRC OX-1 (anti-L-CA) antibody affinity columns and the bound and eluted fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with radiolabeled material visualized by fluorography. The data indicate a complex situation in which all molecules that are reactive with MRC OX-22 antibody are also reactive with MRC OX-1 but not vice-versa. Thus the MRC OX-22 determinant was found on some of the 190,000-, 200,000-, and 220,000-M_r molecules of the L-CA antigen expressed on T cells and on some of the 240,000 M_r L-CA molecules found on B cells. In contrast, the 180,000-M_r form of L-CA on T cells did not react with MRC OX-22 antibody (8, 11).

Functional Studies

Previous studies have shown that T cells that provide help for B cells that are reactive in vivo to allogeneic lymph node cells, that are primarily reactive in mixed leukocyte cultures, and that secrete interleukin 2 (IL-2) on activation are all contained within the CD4$^+$ subset (6, 12, 13). Functional studies were carried out in vivo and in vitro to determine whether the phenotypic heterogeneity of this subset, as revealed by binding of MRC OX-22 antibody (Figure 1 B), correlated with different functional activities.

Alloreactivity of the MRC OX-22$^-$ and MRC OX-22$^+$ Subsets of Inducer/Helper T Cells

In Vivo Studies

The injection of parental T cells into the hind footpads of F_1 hybrid rats produces a gross enlargement of the draining popliteal lymph node within

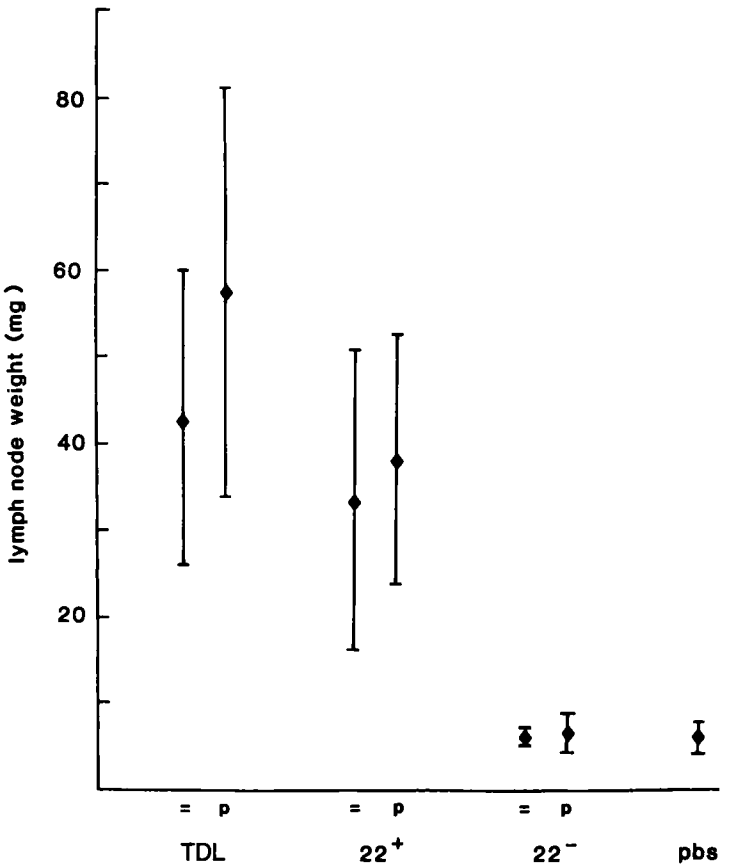


Figure 2. Ability of MRC OX-22$^+$ and MRC OX-22$^-$ subsets of parental strain T cells to mediate popliteal lymph node enlargement in F_1 hybrid recipients. Parental strain TDL, or subsets thereof, were injected into the hind footpads of F_1 hybrid rats and the draining popliteal lymph nodes removed and weighed 7 days later. Two experiments were performed. In the first, the lymph node enlargement induced by 10^7 unfractionated TDL was compared with that obtained with either 10^7 MRC OX-22$^+$ cells or 10^7 MRC OX-22$^-$ cells. The data for this experiment are designated by the equal signs (=) in the figure. In the second experiment "physiologic" cell doses were used, i.e., the numbers of CD4$^+$ MRC OX-22$^+$ and CD4$^+$ MRC OX-22$^-$ cells used were chosen to be equal to those found in 10^7 unfractionated TDL. The results are indicated by *p* in the figure. The negative controls, obtained by injecting medium alone are designated *pbs*. All results are given as means ± 1 SD.

7 days and the degree of node enlargement can be used as a quantitative measure of alloreactivity in vivo (14). As shown in Figure 2, only the MRC OX-22$^+$ cells in parental TDL are functional in this assay, indicating that only CD4$^+$ OX-22$^+$ T cells in rats show this form of alloreac-

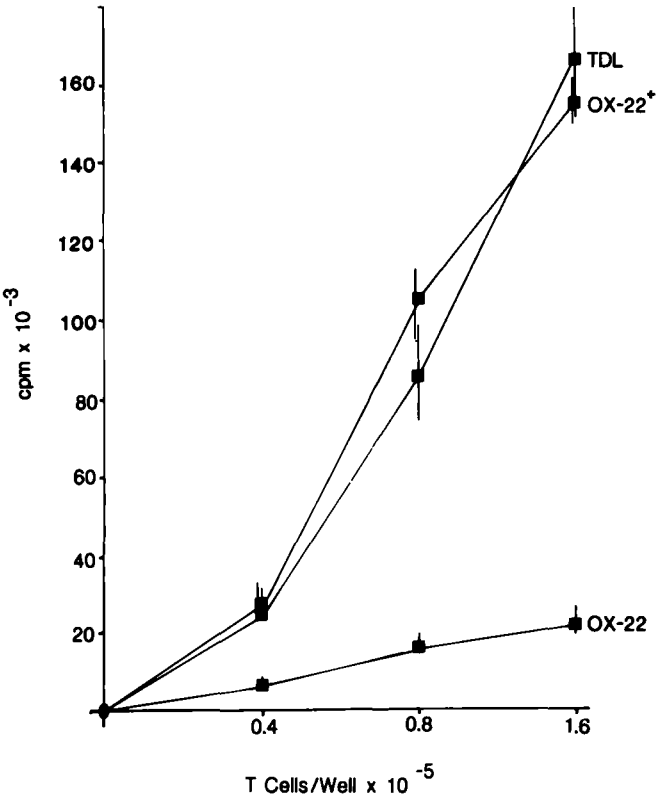


Figure 3. The MRC OX-22 phenotype of the responding T cell in the semiallogeneic MLC. MRC OX-22$^+$ and MRC OX-22$^-$ subpopulations of parental strain TDL were prepared by use of the FACS and cultured with irradiated F_1 splenocytes as stimulators. Unfractionated parental TDL were used as a positive control. The number of parental strain cells added per well were adjusted such that equal numbers of CD4$^+$ T cells were used irrespective of the source of the responding T cells. For example, at the highest concentration tested (1.6×10^5 responder T cells/well), 3.7×10^5 MRC OX-22$^+$ cells, 2.1×10^5 MRC OX-22$^-$ cells, and 3.2×10^5 unfractionated TDL were cultured. [^{3}H]Thymidine incorporation was assayed after 3 days in culture (12).

tivity (6, 8). Similar results were obtained when graft-versus-host disease assays were performed. Previous work has shown that the injection of parental strain CD4$^+$ cells into sublethally irradiated F_1 hybrid rats caused the death of the recipients within 20–50 days (9). When the CD4$^+$ parental cells were fractionated into MRC OX-22$^+$ and MRC OX-22$^-$ subsets, only the former induced weight loss and fatal disease (8).

In Vitro Experiments

The in vitro alloreactivity of various populations of rat T cells was studied by setting up one way mixed leukocyte cultures (MLC) and measuring

cell proliferation and IL-2 production. As shown in Figures 3 and 4, MRC OX-22$^-$ T cells responded very poorly in the MLC and synthesized very low levels of IL-2. In contrast, MRC OX-22$^+$ T cells performed as well as unfractionated T cells in these assays. Combining these results with previous ones on the role of CD4$^+$ and CD8$^+$ cells in these assays (12, 13), it is concluded that the greater part of the alloreactivity of T cells in vitro is mediated by a cell of the CD4$^+$ MRC OX-22$^+$ phenotype, i.e., identical to that showing activity in the in vivo popliteal lymph node assay.

Response of MRC OX-22$^+$ and MRC OX-22$^-$ T Cell Subsets to Concanavalin A

CD4$^+$ cells, isolated from rat TDL, were subfractionated into MRC OX-22$^+$ and MRC OX-22$^-$ populations on the fluorescence activated cell sorter

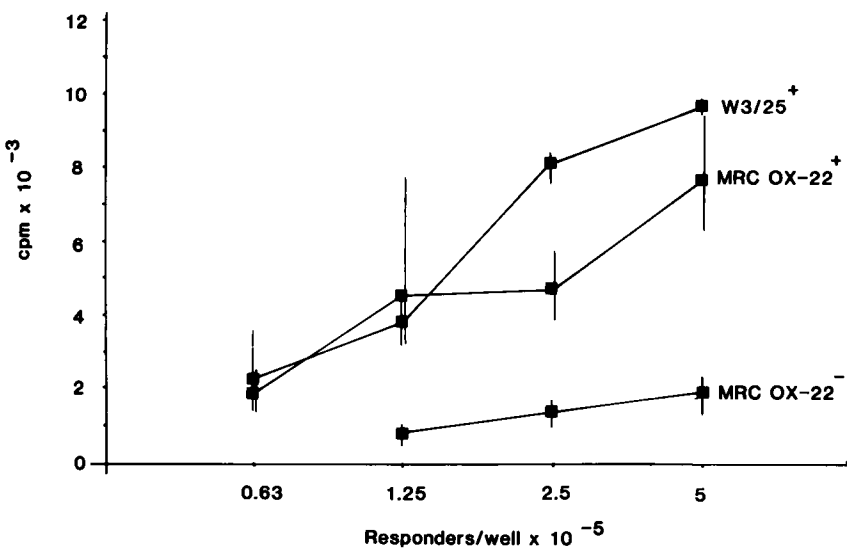


Figure 4. The ability of MRC OX-22$^+$, but not MRC OX-22$^-$, T cells to produce IL-2 in the rat MLC. CD4$^+$ T cells were isolated from parental strain TDL by rosette depletion of sIg$^+$ cells and CD8$^+$ cells. Recovered cells were divided into two portions, one kept on ice and the other labeled with MRC OX-22 antibody and sorted on the FACS into MRC OX-22$^+$ and MRC OX-22$^-$ subsets. All populations were then set up with irradiated F_1 spleen cells as stimulators. Supernatants were removed after 48 hours for the determination of their relative IL-2 content by testing their ability to promote [^{3}H]thymidine incorporation in Con A-stimulated cells (13).*

* The rat CD4 antigen has been termed W3/25 in all the previous literature on T cell antigens in this species. In the present article, the two terms will be used interchangeably.

(FACS), and incubated with concanavalin A (Con A). As shown in Table II, the ability of this T cell mitogen to induce T cell proliferation, as gauged by [^{3}H]thymidine incorporation, was dependent on the presence of accessory cells in the cultures and was almost completely confined to the MRC OX-22$^+$ subset. Similar cultures were assayed for IL-2 production, and, as Table II indicates, MRC OX-22$^+$ cells synthesized much more lymphokine than did MRC OX-22$^-$ ones. As with cell proliferation IL-2 release was dependent on the presence of accessory cells. It

Table II. MRC OX-22$^+$ T Cells, but Not MRC OX-22$^-$ T cells, Respond to Concanavalin A by Proliferation and IL-2 Production

		[^{3}H]thymidine incorporation	
Assay	*Responder cell*[1]	*Without accessory cells*	*With accessory cells*
		cpm × 10^3	
Con A induced proliferation	TDL	7	34
	CD4$^+$	<2	25
	OX-22$^+$	<2	32
	OX-22$^-$	<2	4.5
IL-2 production	TDL	ND	ND
	CD4$^+$	<1	14.5
	OX-22$^+$	<1	11
	OX-22$^-$	<1	<1

CD4$^+$ T cells were isolated from rat TDL by rosette depletion of sIg$^+$ cells, CD8$^+$ cells, and Ia$^+$ cells (to remove the latter population MRC OX-6 monoclonal antibody that reacts with rat Ia (15) was added to the incubation mixture used to label the TDL before rosetting). The CD4$^+$ cells were then labeled with MRC OX-22, sorted into positive and negative fractions on the FACS, and set up in culture with 10 μg/ml Con A. Accessory cells, prepared by rosette depletion of B cells and T cells from rat TDL, were irradiated with 2,000 rads ^{137}Cs irradiation and added to some of the cultures. Proliferation was assessed by [^{3}H]thymidine incorporation after 48 hours in culture and IL-2 production after 24 hours. For the proliferation assay, 2.5 × 10^5 responder T cells/well were used in all cases and, for the IL-2 assay, 0.5 × 10^5 cells/well. Data obtained at the other cell doses showed that neither assay was saturating at the cell doses used here.

Table III. Helper Activity for Hapten-primed B Cells in Vivo Is Provided by MRC OX-22⁻ T Cells

Expt.	*Cells transferred*	*Antibody response as a percentage of control*
1	5×10^6 B cells	0.6
	1×10^7 B + 10^6 OX-22⁻	71
	1×10^7 B + 3×10^6 OX-22⁺	14
	10^7 TDL	100 (control)
2	10^6 OX-22⁻	8.1
	8×10^6 OX-22⁺	11.3
	10^6 OX-22⁻ + 8×10^6 OX-22⁺	138
	10^7 TDL	100 (control)

Donor rats were primed with dinitrophenyl-bovine gamma globulin several weeks before use. Thoracic duct lymphocytes from these donors were labeled with MRC OX-22 antibody and separated into MRC OX-22⁺ and MRC OX-22⁻ fractions. The B cells, which were MRC OX-22⁺, were copurified with MRC OX-22⁺ T cells so that the yield after sorting, of MRC OX-22⁺ cells was approximately eight times that of the MRC OX-22⁻ cells (see Table 1). The cell mixtures, together with 500 μg of dinitrophenyl-bovine gamma globulin, were injected into sublethally irradiated syngeneic recipient rats which were bled 7 days later for assays of anti-dinitrophenyl antibody (8).

is evident that, with respect to the phenotype of the reactive cell, the data obtained with Con A show close similarities to those from the MLC experiments.

Helper Activity for B Cells Is Mediated Predominantly by MRC OX-22⁻ T Cells

In Vivo Studies

Table III (expt. 1) shows the results of an experiment designed to assay the ability of antigen-primed MRC OX-22⁺ and MRC OX-22⁻ T cells to collaborate with hapten-primed B cells in the adoptive transfer of a secondary anti-hapten antibody response. As shown in the Table MRC OX-22⁻ T cells were much more effective than MRC OX-22⁺ T cells in

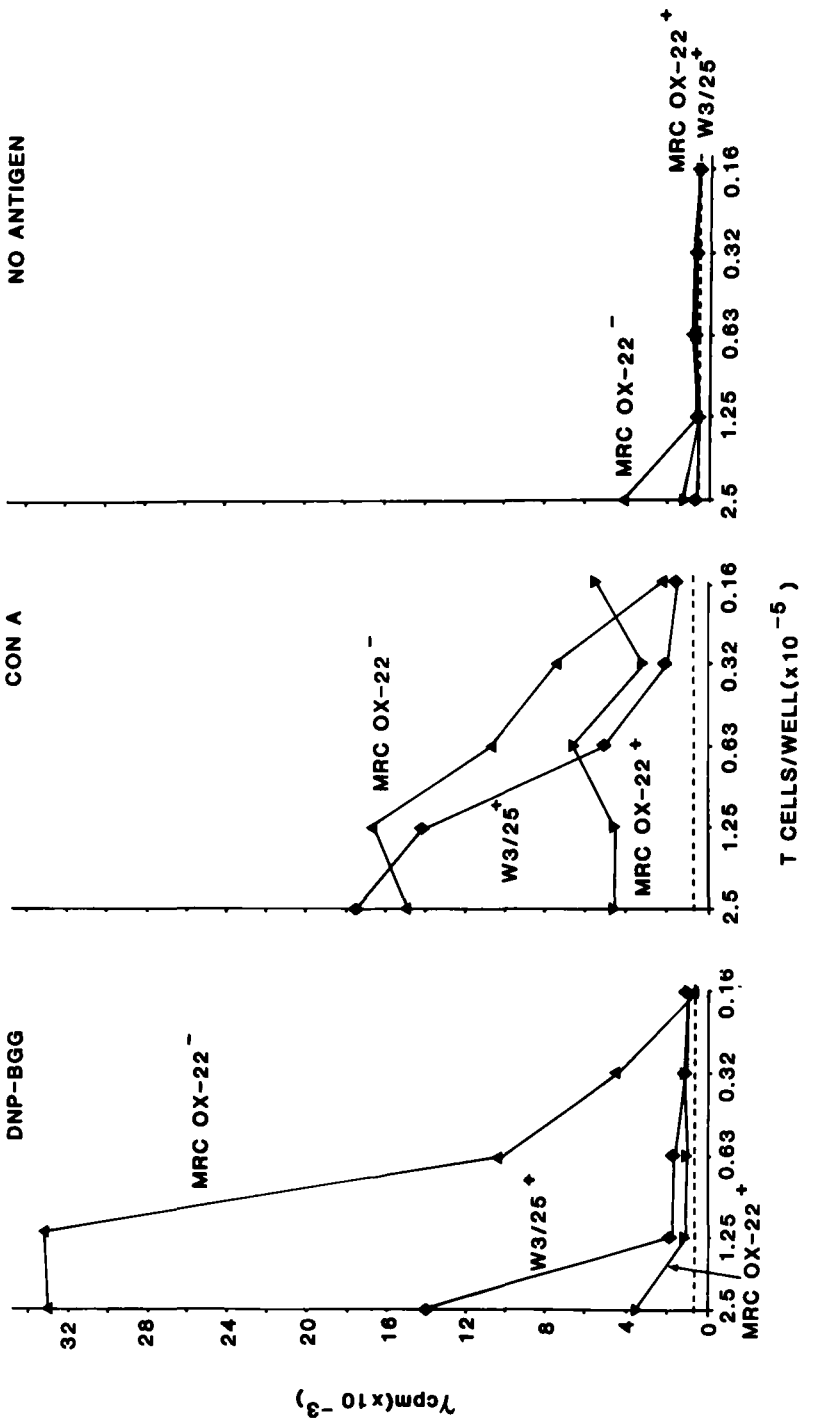
this assay and similar experiments confirmed this result. In particular, the separation of hapten carrier-primed TDL into MRC OX-22$^+$ and MRC OX-22$^-$ fractions demonstrated that neither fraction alone was able to transfer adoptively a secondary anti-hapten response, but that a mixture of the two subsets could do so (expt. 2). An independent experiment established that memory B cells are MRC OX-22 positive so that the observed synergy indicated a helper T cell role for the MRC OX-22$^-$ cells.

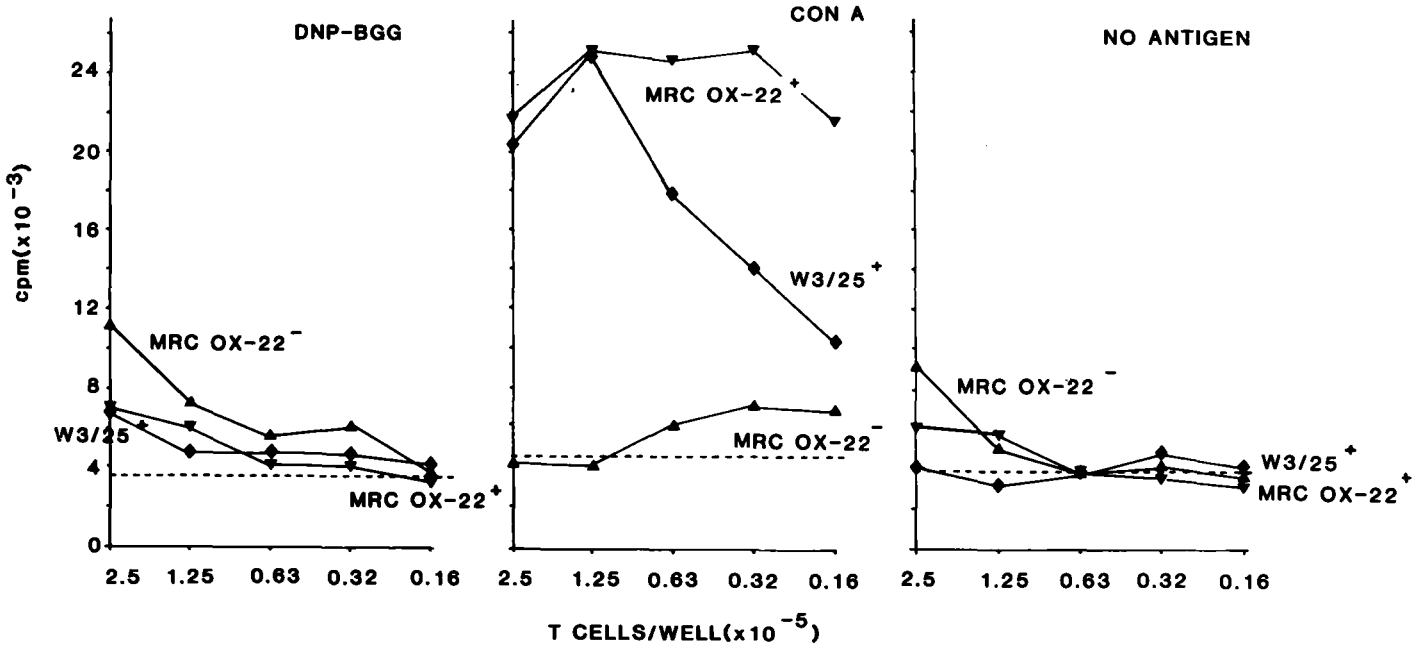
In Vitro Experiments

The in vivo experiments described in the previous section, although indicating the essential role of MRC OX-22$^-$ cells in antibody responses, did not permit studies of lymphokine production in an ongoing secondary B cell response. As the experiments on alloreactivity and Con A responsiveness showed, IL-2 production, in those systems, was predominantly a function of MRC OX-22$^+$ cells. Because IL-2 has been implicated in B cell responses to antigen, either by binding directly to B cells (16, 17) or by inducing T cells to provide B cell help (18), the following experiment was set up to study antibody synthesis and IL-2 release in an in vitro system. A constant number of hapten-primed B cells together with a fixed number of non-B non-T accessory cells were supplemented, in tissue culture, with various T cell subpopulations from antigen-primed donors. To these cultures was added either specific antigen, Con A, or medium alone. After 2 to 3 days of culture small aliquots of supernatant were removed from each well and assayed for IL-2 content and after 6 days in culture all wells were assayed for anti-hapten antibody. As shown in Figures 5 and 6, wells supplemented with MRC OX-22$^-$ T cells and specific antigen produced the highest anti-hapten antibody responses (Figure 5), but little IL-2 (Figure 6), whereas cultures containing MRC OX-22$^+$ T cells produced IL-2 in readily assayable amounts when Con A was added to the cultures, but little or no antibody under any circumstance. The almost complete dissociation between IL-2 production and antibody synthesis was most clearly observed with the Con A cultures where wells containing MRC OX-22$^-$ cells produced antibody but little IL-2, whereas those containing MRC OX-22$^+$ cells yielded high levels of IL-2 but virtually no antibody.

Summary and Conclusions

Two conclusions may be drawn from the data presented in the previous sections, namely, that inducer/helper T cells are functionally heteroge-


DNP-BGG
CON A
NO ANTIGEN
MRC OX-22 −
W3/25 +
MRC OX-22 +
γcpm(x 10 −3)
T CELLS/WELL(x10 −5)
32
28
24
20
16
12
8
4
0
2.5
1.25
0.63
0.32
0.16


DNP-BGG
CON A
NO ANTIGEN
cpm(x10⁻³)
24
20
16
12
8
4
0
MRC OX-22⁻
W3/25⁺
MRC OX-22⁺
MRC OX-22⁺
W3/25⁺
MRC OX-22⁻
MRC OX-22⁻
W3/25⁺
MRC OX-22⁺
2.5 1.25 0.63 0.32 0.16
2.5 1.25 0.63 0.32 0.16
2.5 1.25 0.63 0.32 0.16
T CELLS/WELL(x10⁻⁵)

neous and that in vitro B cell differentiation to antibody-secreting cells can take place without the concomitant production of high levels of IL-2.

The in vivo and in vitro studies of functional heterogeneity produced highly concordant results. Inducer/helper T cells that were MRC OX-22 positive responded to alloantigens in vivo in both the popliteal lymph node assay and the assay for graft-versus-host disease, and it was this subset that proliferated in the MLC and produced IL-2 when cultured with allogeneic cells or with Con A. In contrast, the MRC OX-22$^-$ subset showed no alloreactivity in vivo and very low levels in vitro. It responded poorly to Con A and synthesized little or no IL-2. On the other hand, both in vivo and in vitro, the MRC OX-22$^-$ cells played an essential role in providing help for B cells. No evidence was obtained to suggest that collaboration between the MRC OX-22$^-$ and MRC OX-22$^+$ T cell subsets was required for the development of optimum B cell help.

This latter point raises the question of the role of IL-2 in B cell responses inasmuch as MRC OX-22$^+$ cells were much more effective than MRC OX-22$^-$ cells at producing this lymphokine. However, as Figure 6 shows, culture of antigen-primed MRC OX-22$^-$ cells with specific antigen did appear to yield a just detectable level of IL-2 though the functional importance of this is unknown. However, Figures 5 and 6 (*center panels*) also show that culturing MRC OX-22$^+$ cells with Con A yielded high levels of IL-2 but little antibody, whereas the reverse result was obtained with MRC OX-22$^-$ cells. These data illustrate that IL-2 alone is not sufficient to drive B cells to terminal differentiation and that, if this lymphokine is required (18), then very low levels will suffice. The role of IL-2 in B cell responses is poorly understood at present with no clear concensus view (16–23). However, the present data are clearly in conflict with the report that IL-2 alone is sufficient to induce B cells to differentiate into antibody-secreting cells (24).

Figures 5 and *6*. TDL from rats primed with dinitrophenyl-bovine gamma globulin (DNP-BGG) were used as a source of hapten-primed B cells and hapten carrier-primed T cells. All cell fractionation was carried out by rosette depletion and cell sorting on the FACS. Triplicate cultures containing 10 ng/ml specific antigen or Con A at 10 μg/ml or medium alone were set up with 3.9×10^5 B cells and 1.6×10^4 non-B non-T accessory cells. Unfractionated CD4$^+$ cells, or the MRC OX-22$^+$ or MRC OX-22$^-$ subsets thereof, were added to the B cell/accessory cell mixtures in the doses indicated in the figures. Total culture volumes were 300 μl. Culture wells were assayed for IL-2 content (Figure 6) after 2 days in culture (Con A) or after 3 days (antigen-containing wells and "medium-only" wells), i.e., at times when preliminary experiments showed peak lymphokine titers. Antibody titers (Figure 5) were measured after 6 days of culture in all cases by use of solid-phase radioimmunoassay (8).

The functional assays for B cell help used in these experiments employed antigen-primed cells so that secondary responses were being studied. The assays for alloreactivity and mitogen responsiveness were, conversely, carried out with unprimed cells. The finding that MRC OX-22$^-$ cells were active in assays employing primed T cells, whereas MRC OX-22$^+$ cells functioned in assays where unprimed cells were used could, in principle, be interpreted as indicating that T cells switched from the positive to the negative phenotype on priming. In this view, the expression or nonexpression of the MRC OX-22 antigen would reflect the immune status of a T cell rather than its function. This interpretation can be sustained only if antigen-primed T cells are unresponsive to Con A, and there is no evidence that this is so. Furthermore, thymectomy of young adult rats, although resulting in a modest reduction in the number of peripheral T cells, did not increase the fraction of CD4$^+$ cells that were MRC OX-22 negative, in contrast to what might be expected if they were long-lived memory cells that developed from a nonrenewable source of unprimed MRC OX-22$^+$ precursors (data not shown).

Several monoclonal antibodies have been described that subdivide the inducer/helper T cell subset in man (25–30). Experimental data in humans has, perforce, been confined to in vitro systems, but where comparison is possible, it is evident that the results in rats and humans show similarities. In particular, Schlossman and his co-workers (28, 29) have described two monoclonal antibodies that label two independent subsets of inducer/helper T cells. One of these subsets provides B cell help in a pokeweed mitogen-driven system but responds poorly to Con A whereas the other responds in the converse way. These subsets, therefore, resemble the MRC OX-22$^-$ and MRC OX-22$^+$ subsets, respectively, of inducer/helper T cells of the rat.

There is presumably some functional significance in the heterogeneity of inducer/helper T cells revealed by the present work. The fact that high-level IL-2 production can be achieved in the absence of significant levels of B cell helper activity suggests that cell-mediated immune responses may be regulated independently of antibody-mediated ones. Similarly, inasmuch as potent B cell help can develop with little concomitant IL-2 production, antibody synthesis may procede without the development of cell-mediated immunity. It is well established that cytotoxic T cells (the development of which is almost certainly IL-2 dependent) can be generated against minor transplantation antigens in the complete absence of a humoral response and it is evident that many antibody responses are evoked, for example, to soluble antigens, for which a cell-mediated response cannot occur. The existence of different T cell subsets that pro-

vide help for humoral and cell-mediated responses suggests the possibility of regulating these two arms of the immune response independently.

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Stimulation of the Immune Response: Contributions of Dendritic Cells

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Introduction

Antigen recognition in the immune response is mediated by clonally distinct surface-receptors on lymphocytes. For B cells, the receptors are the classic immunoglobulin heterodimers of H and L chains, and for T cells, the recently discovered α/β or Ti heterodimers (see Palmer, p. 21). The genetic basis for the generation of lymphocyte diversity has been the central concern of immunologists and has to a large extent been clarified. Commitment to a specific antigen occurs when segments of lymphocyte DNA (termed V, D, and J) are rearranged or recombined to form a functional V gene (termed V_κ, V_λ, and V_H in B cells; or V_α, V_β, and V_γ in T cells). Diversity can be generated in many ways: by the existence of multiple V, D, and J segments; by the many combinatorial possibilities among these segments; by somatic alterations at the VD and DJ joining sites; and by somatic mutations in V segments that occur during the maturation of immune responses especially in B cells.

Although lymphocytes are exquisitely differentiated to recognize selected antigens, they are not powder kegs waiting to explode with their function upon antigenic encounter. There are very few examples in which purified *small* B or T lymphocytes are directly stimulated by antigens, mitogens, or antibodies to antigen receptors. Instead, the stimulation of the immune response typically requires accessory or antigen-presenting cells (APC) and helper or inducer T lymphocytes. The ensuing cell–cell interactions and the release of polypeptide mediators (cytokines, lymphokines) then mediate the response.

Acknowledgments

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The requirement for accessory cells became evident in the very first studies of immune stimulation in vitro (1–3). In antigen- and lectin-induced T cell proliferation, and in T-dependent antibody responses, the accessory cells were derived from the plastic- or glass-*adherent* fraction of lymphoid cell suspensions. Adherent populations would function even if exposed to doses of ionizing irradiation or mitomycin *c* that were sufficient to block cell proliferation. Nonadherent cells were the source of responding lymphocytes, and their function was blocked by irradiation or mitomycin *c*. These findings led to the standard approach of studying accessory function—one mixed irradiated, adherent, accessory cells with nonirradiated, nonadherent lymphocytes and then monitored the development of responses in culture over a 2–7-day period.

The requirement for helper or inducer T lymphocytes emerged from studies of B cell responses in situ (4, 5), and later from B cell and cytolytic T lymphocyte (CTL) responses in culture (6, 7). It was observed that the formation of antibodies to foreign red blood cells, and CTL responses in the mixed leukocyte reaction (MLR), were amplified by the addition of helper T cells. In primary responses, irradiated helper cells would not function, but once primed, they functioned normally. To separate helper from effector cells (B lymphocytes and CTL), advantage was taken of the discovery that most helper cells had a distinct phenotype (in mice, Lyt-1$^+$ Lyt-2$^-$ and more recently L3T4$^+$; in rats, W3/25$^+$ OX8$^-$; and in humans T4$^+$ T8$^-$). More recent studies have shown that the distinction of helpers and CTL on the basis of phenotype is not absolute, because Lyt-2$^+$ cells can secrete helper factors and L3T4$^+$ cells can be cytotoxic.

This review considers the role of dendritic cells as stimulators of immune responses. Dendritic cells are a distinct type of accessory cell that were identified when the cellular composition of adherent populations was analyzed (8, 9). Dendritic cells are remarkably potent in activating helper lymphocytes. Here we will (*a*) summarize some of the distinctive features of dendritic cells, especially their stimulatory capacities; (*b*) consider ongoing experiments that are pertinent to mechanism of action; and (*c*) describe available information on the tissue distribution and life history of dendritic cells, including some speculation on possible functions during cell-mediated immunity in situ.

Identification of Dendritic Cells

The first descriptions of dendritic cells were of those in rodent lymphoid organs and afferent lymph (8–14). The cells were larger than lympho-

cytes and very irregular in shape. In the living state, they continually formed and retracted processes. In mouse spleen, dendritic cells were found primarily in adherent fractions. Several properties (Table I) distinguished them from the other firmly adherent cell type—the mononuclear phagocyte. In particular, dendritic cells lacked phagocytic capacities, Fc receptors, and membrane ATPase. Also they became nonadherent cells after overnight culture (Figure 1). Similar observations were found in many other tissues, except that dendritic cells often were nonadherent immediately upon explanation in vitro (10–14).

In all tissues in which dendritic cells were identified, their numbers were small—typically <1% of the total nucleated cells. Techniques were developed to provide populations with 20–95% dendritic cells, depending on the tissue. The enrichment procedures capitalized on such features as low buoyant density in dense albumin, Percoll, or metrizamide solutions; absence of Fc receptors; nonadherence to tissue culture surfaces; and absence of mononuclear phagocyte and lymphocyte surface antigens (20). Purity was monitored by morphologic assays. One counted irregularly shaped cells that were rich in Ia but lacked the markers of other leukocytes. A monoclonal antibody to mouse dendritic cells was developed (clone 33D1) making it possible to selectively deplete this cell type (18, 19).

Table I. Some Distinctive Features of Splenic Dendritic Cells Relative to Macrophages[a]

Nonphagocytic
Fc Receptor negative
Nonadherent after a day in culture
Nonreactive for nonspecific esterase, membrane ATPase, myeloperoxidase
F4/80 antigen − ve; 33D1 antigen + ve
Isolated from white vs. red pulp
High levels of Ia that are not increased by exposure to lymphokines or recombinant interferon

a. See References 8, 9, 15–19.

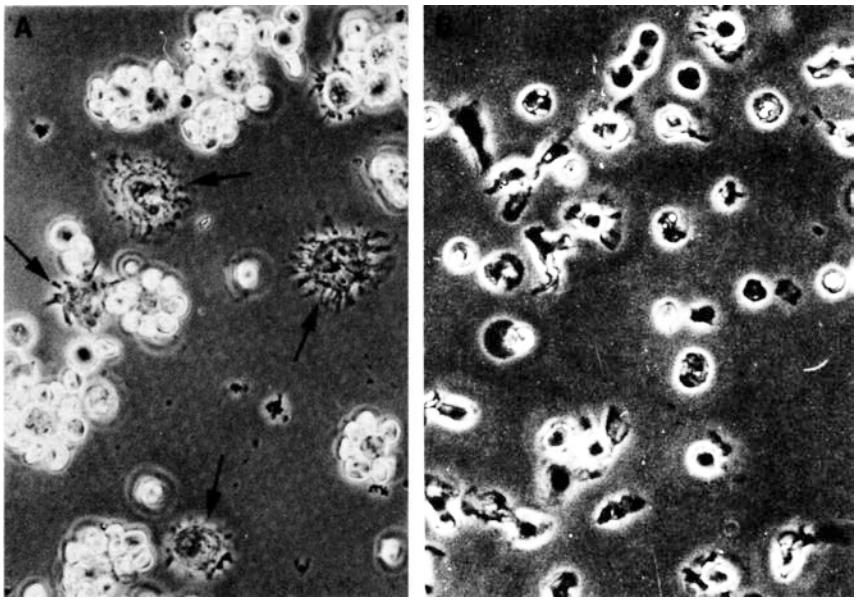

Figure 1. Spleen dendritic cells, phase-contrast microscopy. (A) Spleen adherent cells that have been exposed to opsonized red cells at 4°C and then washed to remove nonbound erythocytes. Note that most cells have Fc receptors and form rosettes, except for the dendritic cells (*arrows*). ×522. (B) Low-density spleen adherent cells after overnight culture. The dendritic cells have become nonadherent and exhibit sheetlike processes or veils. The cultures contain small fragments of cells that were present in the initial adherent fraction. ×119.

Immunostimulatory Capacities of Dendritic Cells

Despite small numbers and difficulties in purification, the stimulatory activities of dendritic cells were repeatedly apparent (Table II). The primary MLR (reviewed in Reference 21) was the first response to be studied and was striking considering the view, at that time, that all Ia^+ leukocytes were active MLR stimulators. Allogenic dendritic cells, which expressed high levels of Ia and H-2, were vigorous stimulators. However, macrophages and B lymphocytes—including B lymphoblasts—were weak or inactive, even though their levels of surface Ia in some instances approached that of dendritic cells. The best example arose when it was discovered that lymphokines induced high levels of Ia in cultured macrophages (16). The macrophages were nonstimulatory in the MLR and did not inhibit the function of dendritic cells. However, as will be discussed later, macrophages and B cells were active stimulators of allogeneic cells that had been *sensitized* by dendritic cells. These MLR findings have important

implications with respect to the mechanism of action of accessory cells. Clearly, antigen presentation (alloantigens on the surface of leukocytes in the MLR) and/or expression of Ia are not sufficient to *initiate* immune responses. Antigen presentation and immune stimulation are not one and the same.

The MLR data had counterparts in models of allograft rejection in situ. Small numbers of F_1 dendritic cells induced rejection of F_1 kidneys that had been rendered nonimmunogenic by parking in immunosuppressed parental hosts (22). Likewise, F_1 dendritic cells induced popliteal lymph node swelling when administered in the footpad of parental recipients (23). In both the kidney and lymph node models, macrophages and lymphocytes did not actively stimulate an allograft response. Faustman et al. (24) implicated dendritic cells in allograft rejection by using both negative and positive selection methods. Treatment of pancreatic islets with 33D1 antidendritic cell antibody and complement allowed for successful grafting across a full major histocompatibility complex (MHC) barrier. Reinfusion of dendritic cells intravenously triggered rejection.

Table II. Responses That Are Stimulated by Dendritic Cells[a]

T cell proliferation
Mitogens: concanavalin A, phytohemagglutinin, $NaIO_4$, neuraminidase/galactose oxidase
Microbial antigens; proteins: Calmette-Guerin bacillus, tetanus toxoid, keyhole limpet hemocyanin (KLH), ovalbumin (OVA)
MLR: syngeneic and allogeneic
Cytolytic T cells
Allo MLR; TNP H-2
T cell-dependent antibody formation
Heterologous red cells
TNP on protein (KLH, OVA) carriers
Allograft rejection
Rat kidney, mouse pancreatic islets, mouse local GVH
Contact sensitivity
Picryl chloride, oxazolone

a. References are provided in Reference 20.

A critical unknown in evaluating stimulatory capacity in situ is the relative ability of different cell types to home to the T-dependent areas of lymphoid organs, where allograft responses are likely to be initiated. There is evidence that dendritic cells are specialized to home to these regions (see below).

Dendritic cells were also essential for primary antibody responses to heterologous red cells and hapten–carrier conjugates (T cell-dependent antigens) in culture (25–28). The early studies of Mitchison (29) and Sprent (30) had shown that B cells presented antigen to sensitized helper T cells. The question therefore arose: if B cells presented antigen, why did one have to add dendritic cells to obtain T cell-dependent antibody responses? Again it appeared that antigen presentation and immune stimulation were not synonymous. Instead there were two stages in an antibody response with distinct accessory requirements (28). First, dendritic cells activated Lyt-2$^-$ helper lymphocytes, and second, the primed helper cells acted directly on histocompatible B cells to induce the antibody response (28). For some antigens, e.g., some epitopes on sheep red cells, a direct B–T cell interaction did not seem necessary. Lymphokines produced by the first dendritic–T cell step bypassed the subsequent need for APC and T cells (26). For other antigens, e.g., hapten–protein conjugates, the second step of B–T cell interaction seemed essential for inducing B cell responsiveness to lymphokines (27).

Mitogen responses have been useful models for documenting early events in T cell proliferation such as the induction of interleukin 2 (IL-2) release and responsiveness. In the system known as "oxidative mitogenesis," T cells entered the cell cycle 24 hours after being modified with sodium periodate or with neuraminidase-galactose oxidase. The "oxidized" T cells required Ia$^+$ accessory cells to grow. Dendritic cells were very active accessory cells, whereas other Ia$^+$ leukocytes were not (10, 11, 31–33). Austyn et al. (32) found that IL-2 progressively accumulated in the culture medium from 3 to 18 hours. If this conditioned medium were removed by washing, and the dendritic cells were killed with anti-Ia and complement, the mitogenesis response ceased. Proliferation resumed with the addition of purified IL-2 or conditioned medium, whereas T cells that had been cultured in the absence of dendritic cells showed little or no IL-2 response. Therefore, coculture with dendritic cells had induced IL-2 responsiveness and release of sufficient amounts of IL-2, prior to entry into the first cell cycle. The accessory cell requirements for other mitogens, such as OKT3 and lectins, still need to be studied to compare dendritic cells with other leukocytes. Mitogenesis to OKT3, a monoclonal to the T3 component of the antigen recognition complex of the T cell, will be of particular interest

because the Fc portion of the monoclonal seems essential. Dendritic cells lack Fc receptors, so they may not be active in the OKT3 response. This in turn indicates that there is no absolute need for dendritic cells in T cell activation.

The study of contact sensitivity illustrated additional features of the function of dendritic cells in situ. Britz et al. (34) found that haptens that were coupled to dendritic cells could induce contact sensitivity even in the presence of a major suppressive stimulus. They administered large doses of trinitrobenzenesulfonate intravenously, which resulted in profound tolerance to subsequent skin painting with picryl chloride. Contact sensitivity could be induced and the tolerance overcome if trinitrophenyl (TNP)-modified dendritic cells were given intravenously. The tolerance to TNP was at least in part attributable to the formation of suppressor cells, so that immunization with dendritic cell-associated antigen dominated over suppression. Knight et al. (35) recently isolated dendritic cells from lymph nodes that drained the site of application of contact allergens. These dendritic cells would induce antigen-specific, T cell-proliferative responses in culture. The conclusion was that contact allergens had become associated with dendritic cells in the draining lymphoid organs, presumably because antigen-laden dendritic cells migrated from skin to node via afferent lymph (see below).

Sensitization of Helper Cells in Culture — Clustering of Dendritic Cells with Responding, Lyt-2⁻ T Lymphocytes

Given the capacity of dendritic cells to initiate T-dependent responses in vitro and in situ, our efforts were directed to an analysis of the sensitization and properties of helper T cells. This became feasible when it was noted that dendritic and helper cells were physically associated in spherical cell aggregates that were 5–20 cells in diameter (Figure 2). Such aggregates formed whenever dendritic cells were stimulating T cells in culture. Virtually all of the viable dendritic cells were in clusters where their sheetlike processes arborized between and around lymphocytes (36).

Experiments in which clusters were separated from nonclusters proved that the aggregates were the site in which dendritic cells primed helper T lymphocytes (26–28, 36). Typically dendritic and Lyt-2⁻ T cells were cultured at a 1:100 ratio for 2 days (Figure 3). Clusters developed and were isolated by velocity sedimentation. Cluster and noncluster fractions (~5–10% and 90–95% of the viable cells, respectively) were then maintained an additional 2–3 days. In the syngeneic MLR (26), clustered T cells proliferated and released such lymphokines as IL-2 and B cell-

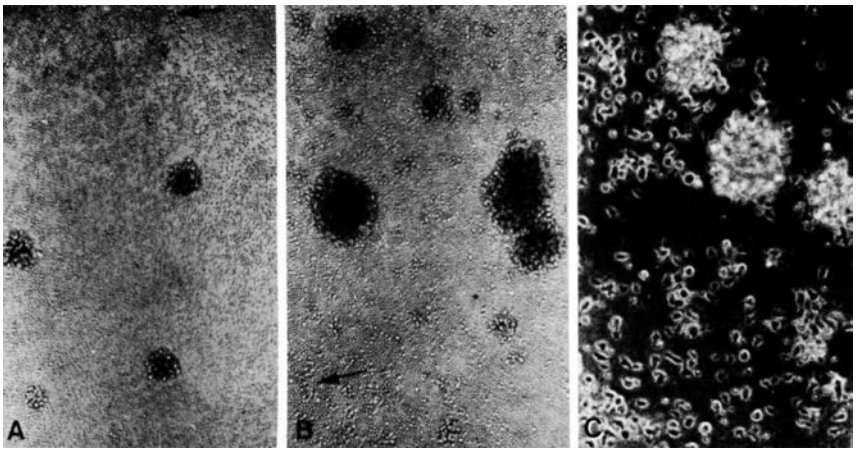

Figure 2. Cell aggregate formation during T-cell dependent responses in vitro. Shown here are primary MLR cultures in which allogeneic dendritic cells have been mixed with Lyt-2$^-$ T cells at a ratio of 1:100. (A) The cultures at day 2. The addition of dendritic cells has led to the formation of cell aggregates, but only a few lymphoblasts are noted (*arrows*). Clusters are not seen when spleen macrophages or B cells are used as APC, but these other APC can enter clusters that have been induced by dendritic cells (27, 28). ×50. (B) The cultures at day 4. The clusters have enlarged and contain many large clear blasts. Blasts are also observed in the nonclustered areas of the culture (*arrows*). ×50. (C) Clusters were isolated by velocity sedimentation from *A* (day 2 MLR) and cultured an additional 2 days. Note the production of large numbers of blasts within and around the aggregates. These released blasts are a source of primed helper cells that are enriched in antigen-specific binding and responsiveness (see text). ×66.

stimulating factors. In the allogeneic MLR (36), the clusters were enriched in specific alloreactivity and depleted of third party reactivity, whereas the nonclustered cells were depleted of a specific alloreactivity and maintained third party responses. In the antibody response (27, 28), most plaque-forming cells developed from the numerically small cluster fraction. Nonclustered cells did not form specific antibody-secreting cells unless supplemented with dendritic and T cells.

Dendritic–T Cell Clusters Generate Primed Helper T Cells

Clusters that were isolated at day 2 of dendritic -T cell cocultures were an excellent source of antigen-specific, primed T cells. When clusters were cultured an additional 2 days (Figure 2 C and Figure 3), lymphoblasts were produced and could be separated from the clusters by a second velocity sedimentation run. Dendritic cells remained within the aggregates. T blasts that had been generated in the allogeneic and syngeneic

MLR, and in cultures primed to protein antigens, were markedly reactive to IL-2 or to specific antigen (Figures 4 and 5). The cell-doubling time was 14–16 hours, and typical tritiated thymidine [^{3}H]TdR uptakes at 24–30 hours were 70–100 $\times$ 10^3 cpm using 3 $\times$ 10^4 starting T cells and standard assay conditions. 3 $\times$ 10^4 blasts gave detectable responses to 30 dendritic cells; optimal responses were seen at 24–30 hours with 300–1,000 stimulators.

If cluster-derived blasts were not restimulated, they quickly ceased growth and release of lymphokines, and they reverted to medium-sized, IL-2 unresponsive "memory" T cells. The memory cells, like the lymphoblasts from which they arose, exhibited highly enriched reactivity to the priming antigen (28). Large proliferative responses began on the second day of culture rather than the first, as was the case with IL-2 responsive lymphoblasts. If helper function was tested, both blasts and memory cells induced maximal antibody responses at T to B cell ratios of 1:100 to 1:1,000 (e.g., Table III).

Therefore, dendritic/Lyt-2$^-$ T cell aggregates are the site in which active helper cells are generated in vitro. The activity of these primed cells equals or exceeds that seen with T cell clones and far exceeds that of populations that are primed in situ. For the latter, one typically administers

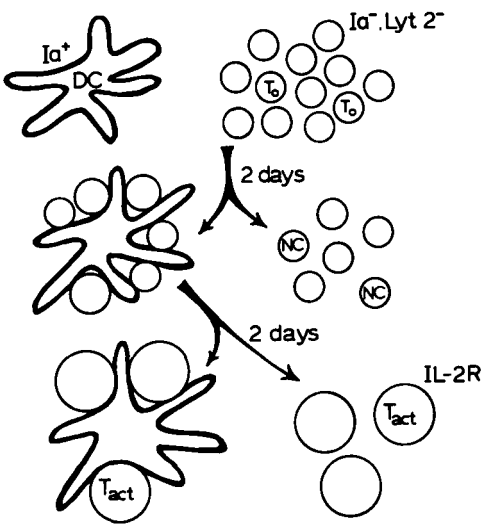

Figure 3. Diagram of the experimental approach to isolating populations of antigen-specific T blasts (T_{act}; see text, Fig. 2, and Reference 28 and 36). Ia$^+$ dendritic cells (*DC*) are cultured with Ia$^-$ Lyt-2$^-$ resting T cells (T_0). After 2 days, dendritic cell/T clusters are separated from nonclusters (*NC*) and cultured 2 more days to produce IL-2 responsive (IL-2R) blasts (Tact).

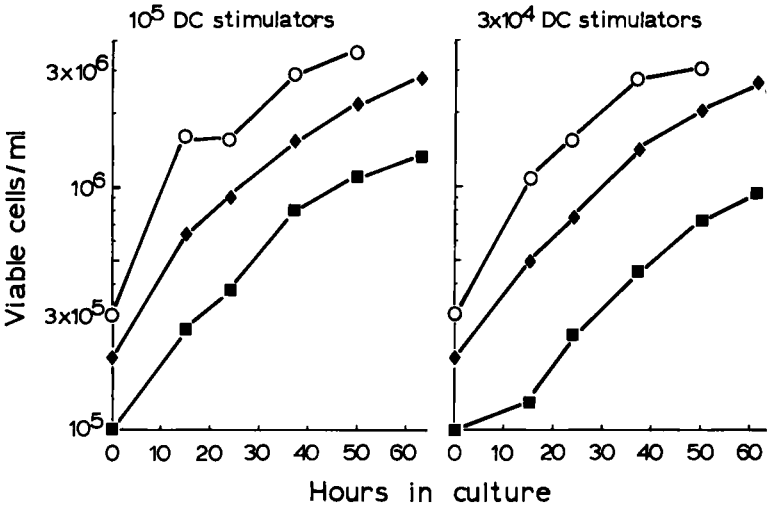

Figure 4. Growth of alloreactive T lymphoblasts upon restimulation with dendritic cells (*DC*) from the original priming mouse strain. The dose of 10^3–10^4 dendritic cells is a saturating dose for the starting numbers of T blasts per culture (1, 2, and 3 × 10^5 blasts in a 16-mm well). Similar initial doubling times are seen if recombinant IL-2 is added (36).

antigen in complete Freund's adjuvant, and the draining nodes are taken at 7–11 days. 3 $\times 10^5$ primed T cells are cultured with equal numbers of irradiated spleen, and proliferative responses are measured at 72 hours. The magnitude of the response typically is similar to that observed when 3 $\times 10^4$ in vitro primed cells are stimulated with 30–100 dendritic cells for 24 hours. Likewise in T cell-dependent antibody responses, in situ primed cells are usually mixed in equal numbers with fresh or primed B cells, whereas in vitro primed blasts or memory cells are added at ratios of 1:100 or 1:1,000. The use of dendritic cells in culture thus provides access to freshly sensitized, oligoclonal populations that are highly enriched in antigen-specific function. This in turn should permit further analysis of T cell priming and the properties of sensitized cells.

Antigen-presenting Cell Requirements of In Vitro-primed T Cells

The first result that was obtained with primed helper T blasts was their distinctive APC requirements. The blasts proliferated and released lymphokines when challenged with antigen presented by dendritic cells, macrophages or B cells. In the primary MLR, only dendritic cells were active. The presenting capacity of macrophages and B cells for T blasts was evident for alloantigens in the allogeneic MLR, self-antigens in the syn-

geneic MLR, and protein carriers (28, 36, 37; Figure 5). Macrophages were almost as effective as dendritic cells, although inhibition of the response was often observed at higher macrophage doses (36); B cells were 5–30 times less effective but clearly active.

The interaction of B cells with T blasts had major consequences with respect to B cell growth and differentiation. B cells were quiescent when cocultured with unprimed T cells but grew and produced antibody in the presence of T lymphoblasts (28, 36; Table III). The B cell plaque-forming cell response to TNP was selected for study, in that T-dependent anti-TNP responses could not be induced de novo by antigen and soluble factors. Instead, a direct MHC-restricted B–T cell interaction was required

Table III. Helper Function of T Blasts Primed to Self (Syngeneic Mixed Leukocyte Reaction) or to Keyhole Limpet Hemocyanin In Vitro

Dose of primed irradiated Lyt-2–T blasts	*Antigen*	*Anti-TNP plaque-forming cells by 2 × 10^6 B + T blasts + antigen (μg/ml)*				
		No Ag	*0.1*	*0.3*	*1.0*	*3.0*
None	TNP-KLH	0	5	5	5	5
10^4 KLH		35	85	159	475	480
3 × 10^4 KLH		30	295	355	665	520
10^5		40	290	690	405	505
10^5 Syngeneic MLR		400	915	1,005	1,155	1,055
None	TNP-HGG	0	—	—	—	—
10^4 KLH		—	0	10	0	10
3 × 10^4 KLH		—	0	0	0	10
10^5		—	25	75	90	65
10^5 Syngeneic MLR		—	515	540	610	575

T blasts were primed in the presence or absence (syngeneic MLR) of 50 μg/ml KLH (28), irradiated, and added to 16-mm wells with 2 × 10^6 B cells (Sephadex G10 nonadherent spleen treated with anti-Thy-1 and Lyt-1 and complement) and graded doses of the indicated antigens. Direct plaque forming cells were measured 4 days later. The anti-TNP response with KLH-primed T blasts was dependent on the linked recognition of hapten (TNP) and carrier (KLH); but with syngeneic MLR blasts, antigen-independent responses were observed (28).

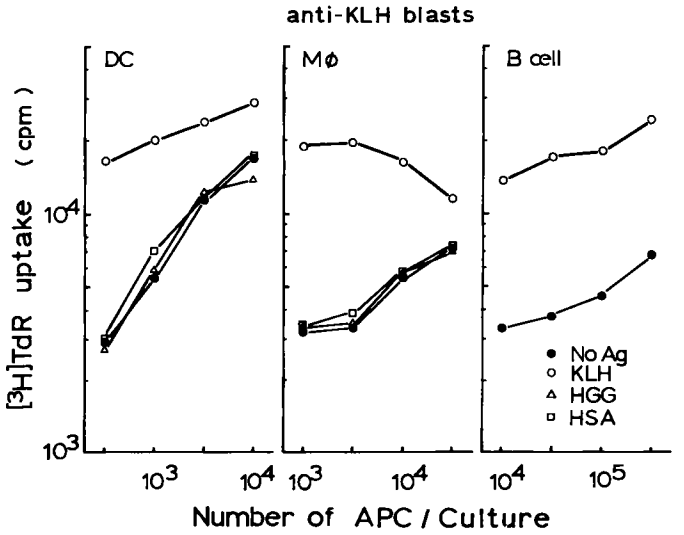

Figure 5. Different types of APC present protein antigens to T blasts. The proliferative responses (cpm [^{3}H]TdR uptake; 1 μCi/0.2 ml culture at 16–24 hr) are from 3 × 10^4 Keyhole limpet hemocyanin (KLH)-primed, Lyt-2$^-$ T blasts (28) restimulated with graded doses of three kinds of APC and the indicated antigens (see key). Dendritic cells (*DC*) are low-density spleen-adherent cells that have been depleted of macrophages; macrophages (Mϕ) are adherent peritoneal cells maintained for 1 day in recombinant gamma-interferon to sustain high levels of Ia; B cells are Sephadex G10-nonadherent spleen cells that are treated with anti-Thy-1 and Lyt-1 and complement to deplete T cells.

and this occurred in cell clusters (27). B cells isolated from the clusters would then mediate an anti-TNP response when maintained in lymphokines, indicating that the B-T cell interaction induced B cell responsiveness to lymphokines (27). It remains to be seen whether direct helper T-effector cell interactions are required for the development of other types of effectors as in macrophage activation and CTL development. These effector cells may respond directly to factors, such as immune interferon and IL-2, and may not require an MHC-restricted helper T-effector cell interaction to become responsive to lymphokines.

We conclude that immune responses can be generated in two phases. In the first phase, dendritic cells initiate T cell growth and lymphokine release. In the second, the sensitized T cells interact with, and/or produce, factors that promote the formation and function of effector cells.

Two Kinds of Interactions between Accessory and T Cells

The interaction between accessory and T cells has always been monitored with functional rather than cell–cell binding assays. The latter would

seem highly desirable as a means of studying the development of the important dendritic-T cell aggregates described above as well as the presentation of antigen to T cells. Binding assays have become possible with the recent availability of in vitro primed T cells. These populations are oligoclonal but highly enriched in antigen reactivity. One can then monitor the binding of bulk T cells rather than selected, chronically stimulated clones. It also appears that the blast is an especially good binding cell in contrast to smaller memory T cells (see below).

Two separate kinds of interaction were identified in which APC and T cells rapidly clustered with one another (36–38). One was antigen-dependent and occurred with many types of APC—macrophages, dendritic cells, B lymphocytes—whereas the other was antigen-independent and occurred primarily with dendritic cells. T cells that were primed to alloantigen, lectin, or to soluble proteins have each been studied. The two types of binding can be illustrated with H-2d anti-H-2k, or alloreactive helper T lymphoblasts. The latter were tagged with carboxyfluorescein diacetate, sedimented on ice with graded doses of APC, and left for 5–10 min on ice or at 37°C. On ice, H-2k but not H-2d dendritic cells (Figures 6 and 7), macrophages, and B lymphocytes all formed clusters

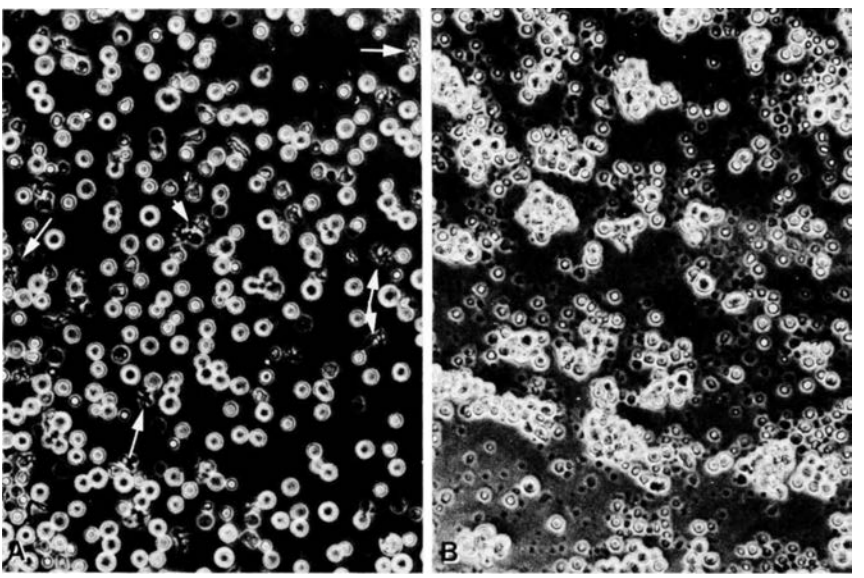

Figure 6. Antigen-dependent and -independent clustering of APC and T blasts. Shown are alloreactive T blasts (H-2d anti H-2k) mixed with syngeneic H-2d (A) or allogeneic H-2k (B) dendritic cells (T to dendritic cell ratio of 3:1) at 4°C. Only allogeneic (antigen-specific) dendritic cells induce T cell clustering at 4°C. Note the nonclustered, syngeneic H-2d dendritic cells at arrows. ×115.

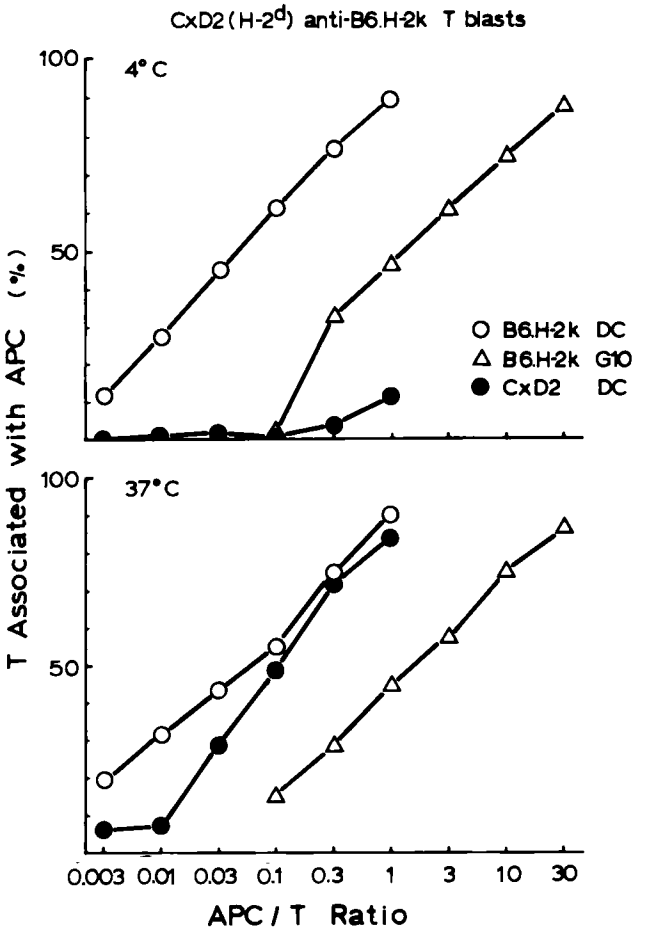

Figure 7. Comparison of dendritic cells (*DC*) and B cells in mediating cluster formation. Graded doses of allogeneic dendritic cells (○) or B lymphocytes (Δ), or syngeneic dendritic cells (●) were mixed with alloreactive T blasts. Note that dendritic cell clustering is antigen-independent at 37°C (●). Syngeneic B cells were not tested, but in other experiments, B cells do not form antigen-independent clusters.

although the dendritic cells were the most efficient (38). At 37°C, macrophages and B cells (Figure 7) bound to T cells with efficacy equal to that seen at 4°C. However, dendritic cells exhibited an antigen-independent interaction at 37°C such that H-2d dendritic cells actively clustered the T blasts (Figure 7). The dose of dendritic cells that was required to see the latter phenomenon was small (at a dendritic cell to T ratio of 1:30 to 1:100, >20% of the T blasts were clustered). Even much higher doses of B cells or macrophages produced little or no antigen-independent

binding (38). Another example was provided by polyclonal concanavalin A-induced T blasts that efficiently clustered with both syngeneic or allogeneic dendritic cells again at 37°C but hardly at all at 4°C. Both memory and freshly sensitized T cells (Lyt-2^+ and Lyt-2^-) actively bound to dendritic cells during antigen-independent clustering, whereas unprimed populations were some 10 times less active. This feature of memory T cells may contribute to the enhanced responsiveness that is observed with primed populations (28, 37).

Green and Jotte (39) have also reported antigen-independent clustering in primed rat T cells. Dendritic cells were markedly more active in inducing aggregation than macrophages and B lymphocytes. Blocking studies with anti-rat monoclonal antibodies indicated that Ia on dendritic cells, and W3/25 (the rat CD4) on T cells, both contribute to the clustering event.

The mechanism and consequences of the two types of clustering can be discussed, but more work is needed. The antigen-dependent interaction, which occurs between most APC and sensitized T blasts at 4°C, may be mediated simply by the binding of "presented" antigen on the APC to the T cell receptor for antigen and MHC. This interaction may be all that is required during the "effector" limb of cell-mediated immunity in which primed T cells stimulate APC (macrophage, B lymphocyte, endothelial cell) directly and/or release important lymphokines. The antigen-independent interaction may require T4 on T cells and Ia on dendritic cells, as suggested by Green and Jotte (39). However, this may not be a simple ligand–receptor interaction, because it does not occur at 4°C, and it does not readily explain the requirement for Ia^+ dendritic cells as opposed to other Ia^+ APC. Although these clusters disassemble quickly at 37°C (28), the antigen-independent component may synergize with the antigen-T cell receptor interaction to initiate the immune response and to maintain the activated state in primed T cells. One could envisage that the antigen-independent pathway provides a separate signal, or simply prolongs presentation and cytokine function.

Although we have emphasized the direct study of cell–cell interactions, there are many other variables — primarily soluble mediators — that need to be added to the accessory-T cell equation and that have not been considered here. These include "activating" factors such as interleukin 1 (IL-1), the trophic functions of accessory cells that contribute to lymphocyte viability and growth particularly in mice, and suppressor factors such as the prostaglandins. All of these factors may have distinct cells of origin and targets, and need to be studied with defined, primary APC populations. We think that the study of immune stimulation must expand beyond

its traditional confines of "processing and presentation" and consider in more detail the physiology of the individual presenting and responding elements.

Epidermal Langerhans Cells

The epidermis of all mammals contains an Ia^+, bone marrow-derived, dendritic cell system (40, 41). These Langerhans cells (LC) are considered here because they are providing important new leads on the origin and function of dendritic cells. Although it had been evident for some time that LC were Ia^+ leukocytes (40, 41), their precise lineage was uncertain. Langerhans cells were very similar to lymphoid dendritic cells by cytologic criteria (42–44). The nucleus was very irregular in shape, and the cytoplasm was electron lucent with few ribosomes, lysosomes, or evidence for phagocytic activity. A distinction was the LC or Birbeck granule, a "tennis racket"-shaped organelle that was not found in most populations of lymphoid dendritic cells. In addition, a detailed analysis by Romani et al. (45), using electron microscopy to identify LC and two-antibody immunofluorescence to phenotype the Ia^+ epidermal cells, showed that most if not all LC express several markers that are found on mononuclear phagocytes but not lymphoid dendritic cells (Figure 8). These markers were the F4/80 antigen, Fc receptors, and membrane ATPase and nonspecific esterase.

A fascinating picture then emerged when LC were isolated and studied in culture (42, 43; Table IV). Most macrophage-like traits were lost including Fc receptors, the F4/80 antigen, and cytochemical markers.

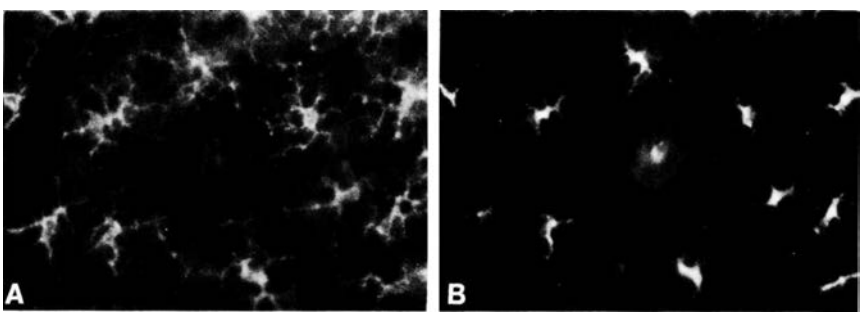

Figure 8. Immunolabeling of murine epidermal sheets. The same specimen is shown stained with F4/80 rat anti-mouse macrophage monoclonal antibody using a triple-layer method (A) followed by fluorescein-conjugated B21-2 anti-Ia monoclonal antibody (B). The method is described in Reference 42. Note that F4/80 and Ia antigens are expressed by the same dendritic cell population. ×48.

Table IV. Properties of Epidermal Langerhans Cells in Culture[a]

	Langerhans cells	
Parameter	*Fresh (7h)*	*Cultured (3d)*
Birbeck granules	+	–
Surface constituents		
Ia	3–4 × 10^5 sites	4–6 × 10^5 sites
Mac-1 (C3biR)	+	+
Fc receptors	+	–
F4/80 Mac Ag	+	–
Cytochemistry		
Nonspecific esterase	+	–
Membrane ATPase	+	–
Stimulation of the MLR and oxidative mitogenesis	Weak	Strong (↑30-fold)

a. See References 42–44.

The LC were nonadherent and looked identical to cultured spleen dendritic cells (Figure 9). Langerhans cells did not increase their levels of Ia in response to immune interferon, and did not internalize tracers such as horseradish peroxidase, nor the debris and dead cells that were in the epidermal cultures. However, LC may be capable of some endocytic activity, because some pinocytosis in situ and phagocytosis in vitro has been observed (46, 47).

When immunostimulatory properties were assessed, the LC proved to be weak stimulators of T cell proliferation, as in the MLR and in oxidative mitogenesis (42, 43). Only upon culture did stimulatory capacity progressively but impressively increase. After 3 days, LC were even more active than spleen dendritic cells. Recent work shows that the weak stimulatory activity of fresh LC is not due to a deficiency in antigen presentation, i.e., the capacity to react with T cells in an antigen-specific way. Freshly isolated LC can stimulate *primed* T lymphoblasts (Figure 10). In clustering assays, fresh LC exhibit antigen-dependent but not antigen-independent binding (Figure 11). The thrust then is that LC are immature dendritic cells that express Ia and present antigens; however, LC

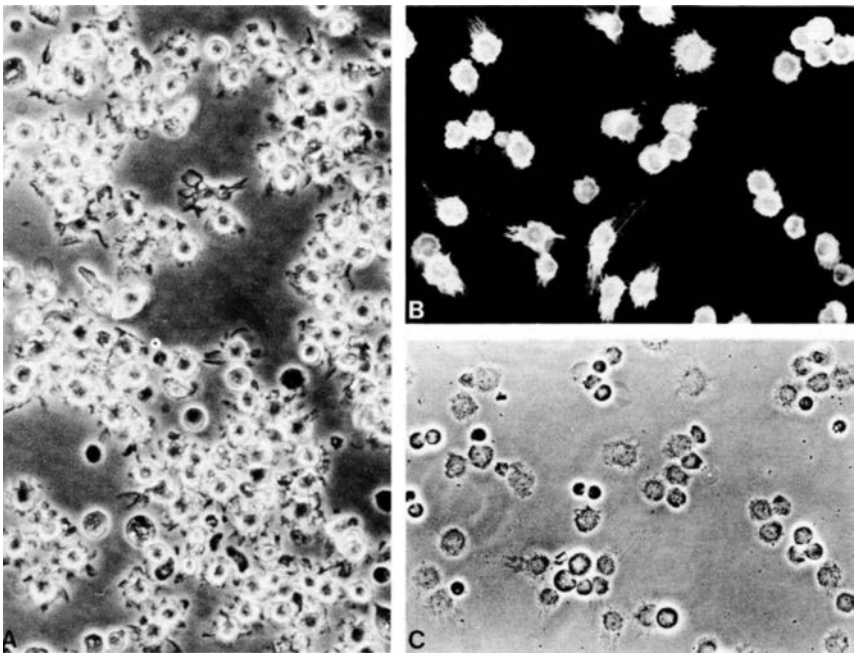

Figure 9. Features of cultured Langerhans cells (*LC*). (A) A 3-day culture of nonadherent, low-density, epidermal cells containing a majority of veiled or dendritic LC (phase-contrast at ×156). On the right is a cytospin preparation of a similar culture (>50% LC) as seen by phase contrast (C) and immunolabeling with anti-Ia (B, ×180). Note the many irregularly shaped LC by phase contrast, which are easily distinguished from the round, Ia^-, contaminating keratinocytes. The fine Ia^+ processes typify cultured LC and spleen dendritic cells.

lack the antigen-independent clustering capacity and do not initiate primary responses like the MLR and antibody formation.

Origin and Tissue Distribution of Dendritic Cells

A bone marrow origin for dendritic cells and LC has been established (48–50), but their relationship to other marrow-derived lineages is unclear. Ongoing studies (Schuler, Van Furth, Witmer, and Steinman, unpublished data) show that few if any dendritic cells develop when mouse marrow is cultured in a fibroblast-conditioned medium that supports the growth and differentiation of large numbers of monocytes and macrophages. Other studies indicate marked differences in the phenotype and function of dendritic cells and monocytes in mouse and in humans (17, 51). However, LC give rise to typical dendritic cells in culture but also

express several myeloid features, such as the mac-1 antigen of macrophages and granulocytes and the F4/80 antigen of macrophages (but not many monocyte-specific antigens, e.g., Reference 51). The data base is obviously small, but our working hypothesis is that dendritic cells are myeloid elements that become committed to a distinct differentiation pathway in the marrow. The marrow emigrant may then give rise to dendritic cells in tissues, such as epidermal LC or the dendritic cells in pancreatic islets, or they may seed lymphoid organs like spleen directly. Small numbers of dendritic cells have been identified in human blood (51).

In the lymphoid tissue, the T cell-dependent regions contain Ia-rich "interdigitating" cells (Figure 12). These correspond to the dendritic cells that are isolated from lymphoid organs in cytologic features and expression of abundant Ia (52, 53). Some interdigitating cells exhibit LC granules (54), but markers such as F4/80 (antimacrophage), 2.4G2 (anti-mouse Fc receptor), and 33D1 (antidendritic cell) have not been detected (52, 55). There is a possibility that the dendritic cells that are released from lymphoid organs are not a full sample of "interdigitating cells." This concern arises because lymph nodes are the organs that are richest in inter-

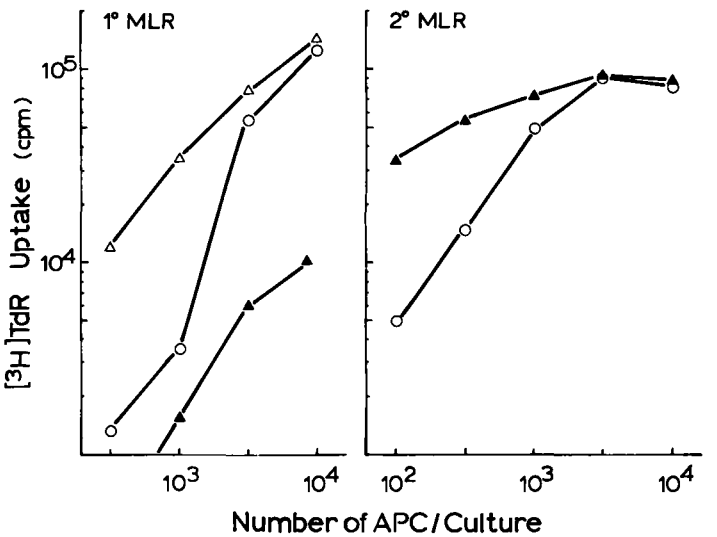

Figure 10. Freshly isolated Langerhans cells (*LC*) actively stimulate alloreactive T blasts in the secondary (2°) MLR, but not unprimed T cells in the primary (1°) MLR. Langerhans cells (▲) were enriched to 50% purity by killing most keratinocytes with anti-Thy-1 and complement. These were compared with spleen dendritic cells (○) and with enriched (5%) 3-day cultured LC (△). The responses were 3 × 10^5 and 3 × 10^4 unprimed and primed Lyt-2⁻ T cells. [^{3}H]TdR uptakes were at 72–90 hours in the primary MLR, and 18–24 hours in the secondary MLR.

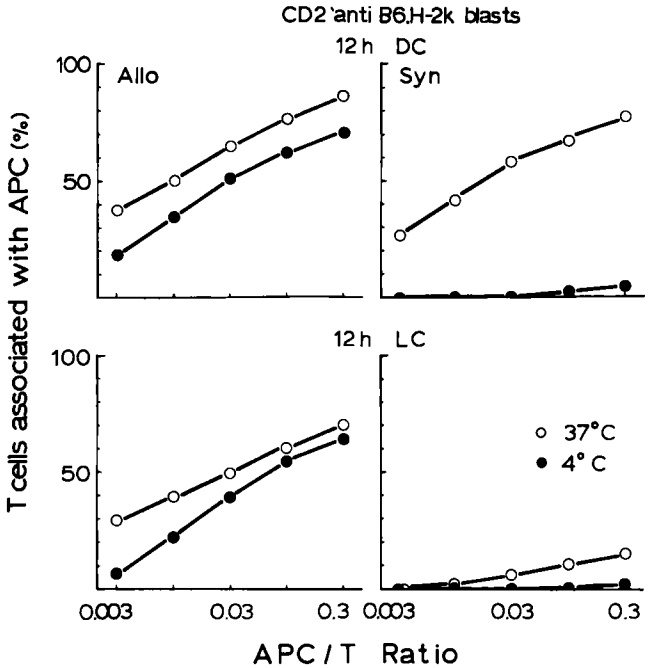

Figure 11. Freshly isolated Langerhans cells (*LC*) (12 hours after trypsin dissociation of epidermal sheets, Reference 42) present antigen in binding assays. This experiment is redrawn from Figure 11 in Reference 38. Note that spleen dendritic cells (*DC*) and LC both bind in an antigen-specific fashion to alloreactive (*Allo*) T blasts. However, only the dendritic cells show antigen-independent (*Syn*) binding at 37°C.

digitating cells, and yet dendritic cells are only released in small numbers relative to spleen (8).

The "veiled" cells in afferent lymph closely resemble lymphoid dendritic cells. Ia is abundant; morphologic features are similar; Fc receptors and phagocytic activity are not apparent; and T cell stimulatory capacities are clear (12–14, 23, 49). Some veiled cells contain LC granules, and their frequency seems to expand during epidermal contact sensitivity reactions (54, 56, 57). Therefore it has been proposed that contact sensitivity reactions are associated with a flux of antigen-laden LC up the afferent lymph and into the T areas of the draining lymph node.

Recently, dendritic cells have been identified in large numbers in the inflammatory exudates of rheumatoid arthritis (58). It is not clear whether these dendritic cells are derived from the blood, or represent a reflux of cells of the Langerhans type trying to gain access to the afferent lymph. In other inflammatory exudates, such as those induced by thioglycolate in the mouse peritoneal cavity, it has been difficult to demonstrate

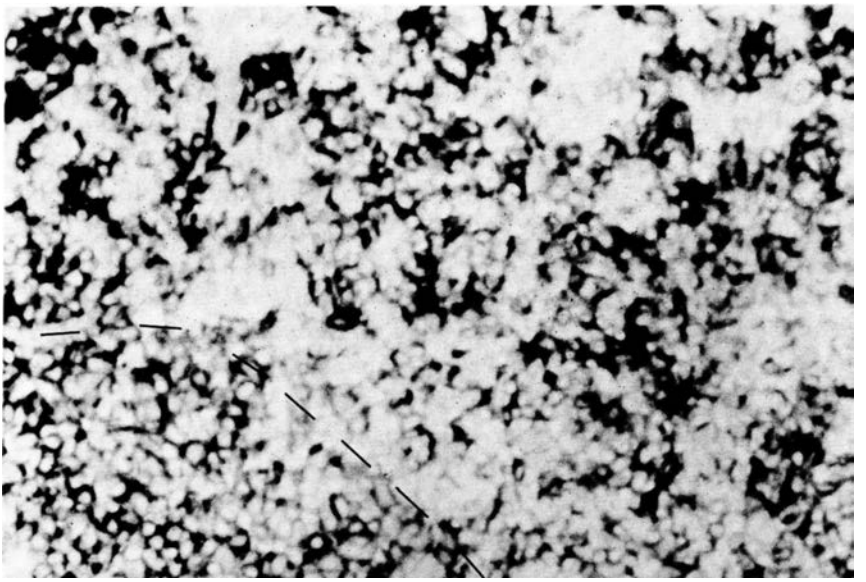

Figure 12. Interdigitating cells in the T cell dependent areas of peripheral lymphoid organs. Sections of mouse spleen were stained for Ia antigens using the B21-2 monoclonal and peroxidase immunocytochemistry (see Reference 52). Note the follicle of round B cells on the lower left (*dashed line*) and the scattered Ia-rich dendritic profiles in the periarteriolar sheath (T cell-dependent area). ×37.

significant increases in the number of dendritic cells (8). Current data on the tissue distribution of dendritic cells are summarized in Figure 13.

Possible Role of Dendritic Cells in Cell-mediated Immunity

We began this chapter by emphasizing the capacity of T cells to respond to a wide variety of antigens. Another important feature is that the system must sense antigen and mount an effective response wherever antigen is deposited. Although systemic activation of host resistance occurs with

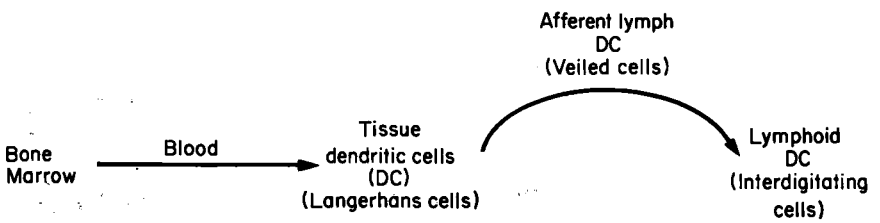

Figure 13. Diagram indicating the tissue distribution of dendritic cells (DC) according to current evidence (see text).

macrophages during infection with some facultative organisms (59), optimal resistance may require the mobilization of both APC and specific lymphocytes to the tissue site where antigen resides. To understand this "mobilization" feature of cell-mediated immunity, it will be important to identify the locales in which immunostimulatory dendritic cells function.

The first unknown is the site in which antigen primes specific T cells. The initial sensitization may not occur locally at the site of antigen deposition but may primarily occur in the spleen or lymph nodes that receive blood and afferent lymph draining the site of antigen deposition. A classic example is the experiment showing that the development of skin allograft immunity is blocked if the afferent lymphatics that drain the graft are severed (60). In most T cell-dependent responses in situ, the draining lymphoid tissue is a rich source of sensitized cells. One could make a strong argument to support the need for draining lymphoid organs at the onset of the response. Lymphoid organs allow for the continued recirculation of lymphocytes, making it possible for antigen to encounter the infrequent clone that is committed to recognize that antigen. It is not yet evident that a corresponding criculation of lymphocytes could occur in an inflammatory site. Lymphoid tissues also contain several types of nonlymphocytic cells (mature dendritic cells, follicular dendritic cells, and macrophages; see Reference 52) that function in antigen stimulation, retention, and clearance, respectively. In contrast, nonlymphoid tissues may not contain immunologically active dendritic cells. Therefore the draining lymphoid tissue is the logical site where antigen, accessory cells, and antigen-specific lymphocytes act together to generate an immune response as was first demonstrated 50 years ago by McMaster and Hudak (61).

Although the numbers of dendritic cells that are isolated from lymphoid tissues seem small, the supply could well meet the demand. The interdigitating cells in T cell areas (Figure 12) seem numerous relative to the numbers of clones that need to be stimulated in the immune response. The size of dendritic cell tissue reservoirs, as well as the capacity to be mobilized into the lymph, also could be substantial. For example, the number of epidermal LC is some 20 times the number of spleen dendritic cells (42), and the flux of dendritic cells into lymph can be large (10^5 cells/hr in rat mesenteric afferent lymph [49]). The inflammatory response and necrosis that accompanies antigen entry could also be critical in the onset of dendritic cell function. Tissue dendritic cells may be mobilized into the lymph, their functional maturation may be induced, or they could pick up the antigen and move with it to the T areas of lymphoid tissue.

Another event that occurs in the draining node during the immune response is the emergence of large numbers of lymphoblasts via the efferent lymph (62, 63), and from there into the blood and eventually to the inflammatory site. Presumably these blasts are produced in dendritic cell T cell clusters as occurs in vitro. Sensitized cells might then accumulate in inflammatory sites in an antigen-independent fashion (64, 65). We would assume that antigen presentation to the blasts could be accomplished by many types of APC (macrophages, B cells, tissue dendritic cells, and Ia^+, endothelial cells), inasmuch as this is what is observed in culture. However, it is important to point out that short-term responses are measured in culture, and that freshly sensitized blasts are required as the responding cells. It may be that the presence of dendritic cells in the tissues could allow for prolonged local responses and/or activation of memory lymphocytes.

This view of the generation of cell-mediated immune responses obviously is made to accomodate as much of the tissue culture data as possible. Although a good deal has been learned about the types and mechanism of action of APC in vitro, relatively little is known about the distribution of these cells and antigen during cell-mediated immunity in situ. There would seem to be enough information on dendritic cells, including experiments in situ (22–24, 34, 35) to warrant more direct work in whole animal models. One can propose, for example, that antigen in association with dendritic cells will provide an effective means of inducing host resistance. Elimination of dendritic cells may reduce the barriers to transplantation. Techniques for mobilizing and maturing tissue dendritic cells could provide avenues for immunotherapy.

Summary

Dendritic cells occupy a special place among APC in view of their potent stimulatory activities, particularly for unprimed and memory T cells. Some examples of responses that are initiated by dendritic cells are: MLR in vitro and allograft responses in situ; primary antibody responses to T cell-dependent antigens; production of lymphokines; and contact sensitivity reactions in situ. In contrast, other APC such as macrophages and B lymphocytes act on freshly primed T cells that have been sensitized by antigen and dendritic cells. During immune responses in culture, dendritic cells cluster with the responding T lymphocytes. Clustering can occur by separate antigen-dependent and -independent pathways. The latter pathway seems qualitatively restricted to dendritic cells and may underlie their stimulatory properties. The origin and life history

of dendritic cells is being characterized and likely includes stages in which they occur in tissues (Langerhans cells), afferent lymph (veiled cells), T-dependent zones of lymphoid organs (interdigitating cells), and some inflammatory exudates (rheumatoid arthritis). During cell-mediated immunity, dendritic cells may play important roles in the delivery of antigen to lymphoid organs, in the generation of sensitized T lymphoblasts in the T cell-dependent regions, and in the elicitation of T cell function in the inflammatory site.

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Response of Purified T Cell Subsets to H-2 Alloantigens

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Introduction

Cell-mediated lympholysis (CML) and the mixed-lymphocyte reaction (MLR) are the most popular in vitro assays for measuring the response of T cells to allogeneic gene products of the major histocompatibility complex (MHC), the H-2 complex in mice. It is generally accepted that the cells participating in these two assays are largely nonoverlapping. Most, though not all, T cells exerting CML have specificity for class I (H-2K, H-2D) H-2 alloantigens and express the Lyt-2$^+$, L3T4$^-$ phenotype (1–3). The induction and differentiation of cytotoxic T lymphocyte precursors (CTLp) is held to require interaction with the other subset of T cells, i.e., Lyt-2$^-$, L3T4$^+$ T helper (Th) cells (1–5). These cells are specific for class II (I-A, I-E) (Ia) H-2 alloantigens and exert strong MLR. Interleukin 2 (IL-2) released by Th cells amplifies the growth of CTLp and controls their differentiation into cytotoxic cells.

Although the view that MLR are directed predominantly to class II rather than class I alloantigens is now dogma (6), it tends to be forgotten that certain class I differences, particularly mutant class I differences, can induce appreciable MLR (7–9). Although the existence of anti-class I MLR might suggest that some T cells specific for class I molecules are helper-independent, it has been argued that these responses are in fact controlled by the Th subset recognizing the allo class I molecules in association with self-class II molecules (8, 9). Such findings reinforce the view that, in contrast to class II-restricted cells, the differentiation of class I-restricted T cells depends crucially upon exogenous help provided by other cells. An obvious corollary to this viewpoint is that purified popu-

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lations of unprimed CTLp, i.e., purified Lyt-2$^+$ cells, would be unable to generate MLR or CML unless supplemented with Th cells or their products. In this chapter, I will summarize recent studies from this laboratory which indicate that, contrary to popular opinion, purified Lyt-2$^+$ cells do give high primary MLR and CML responses to class I differences in the absence of exogenous help.[1]

Preparation of Purified T Cell Subsets

Experiments designed to determine whether isolated Lyt-2$^+$ cells can respond to alloantigens obviously depend on preparing these cells in a highly purified form. We use a two-step procedure for cell purification. First, lymph node (LN) cells are treated in the presence of C′ with a monoclonal antibody (MAb) specific for the L3T4 molecule (GK1.5) (2). The cells are treated simultaneously with an anti-B cell MAb, J11d (10); this antibody has no specificity for mature T cells. Cells surviving treatment with anti-L3T4 and J11d MAb + C′ are then placed on plastic dishes coated with anti-Lyt-2 MAb. After incubation for 45 minutes at 4°C, nonadherent cells are carefully washed off the dishes. The adherent cells are then eluted by vigorous pipetting with a Pasteur pipette. By fluorescence-activated cell sorter analysis these cells are >99.5% Lyt-2$^+$ and contain no detectable L3T4$^+$ cells. An analogous procedure is used to prepare L3T4$^+$ cells, i.e., pretreating LN cells with anti-Lyt-2 and J11d MAb + C′ followed by panning on anti-L3T4-MAb-coated plates.

Response of Purified T Cell Subsets in Mixed-Lymphocyte Reactions

Mutants of the C57BL/6 (B6) (H-2^b) strain are an invaluable aid for studying MLR directed selectively to class I or class II differences (11). The B6.C-H-2^{bm1} (bm1) mutant strain is identical to the wild-type B6 strain except for three amino acid substitutions in the H-2K molecule. The class II mutant B6.C-H-2^{bm12} (bm12) differs from B6 by three amino acids in the β-chain of the I-A^b molecule. Mixed-lymphocyte reactions of B6 cells to bm1 and bm12 stimulators are thus directed exclusively to allo class I and allo class II molecules, respectively.

Mixed-lymphocyte reactions mediated by L3T4$^+$ cells show several characteristic features (Table I). First, the background counts observed with

1. The in vitro data summarized in this paper will appear in: Sprent, J. and M. Schaefer. 1986. Properties of purified T cell subsets. I. In vitro responses to class I vs. class II H-2 alloantigens. *J. Exp. Med.* In press.

Table I. Mixed Lymphocyte Reactions of B6 L3T4⁺ and Lyt-2⁺ Cells to Whole H-2 Differences and Class I vs. Class II H-2 Differences

Responders (1×10^5)	*Stimulators (anti-Thy-1 + C'-treated)*	*Stimulus*	*[³H]TdR incorporation*	
			Day 4	*Day 6*
			cpm $\times 10^{-3}$	
B6 L3T4⁺	B6	–	1.0	2.3
	(B6 × CBA/Ca)F_1	H-2^k	41.6	145.0
	(B6 × bml)F_1	K^{bml}	3.3	6.1
	(B6 × bml2)F_1	I-A^{bml2}	25.4	98.2
B6 Lyt-2⁺	B6	–	0.2	0.1
	(B6 × CBA/Ca)F_1	H-2^k	84.6	1.7
	(B6 × bml)F_1	K^{bml}	99.7	2.4
	(B6 × bml2)F_1	I-A^{bml2}	0.8	2.5

Cells cultured in RPMI 1640 with 10% fetal calf serum; stimulator cells (5×10^5) received 1,500 rads. The data represent mean of triplicate cultures; standard deviations were generally within 10% of the mean.

syngeneic stimulators (auto MLR) tend to be higher than with unseparated T cells (Table I and unpublished data). Second, the responses increase progressively with time, peak responses being observed on day 6 (the latest time point examined). Third, significant responses are observed only to class II and full H-2 differences and not to class I differences.

As shown in Table I, B6 Lyt-2⁺ cells give surprisingly high primary MLR. The following features characterize the response of Lyt-2⁺ cells. First, auto MLR by Lyt-2⁺ cells are usually almost undetectable. Second, the allo response of Lyt-2⁺ cells peaks quite early, usually on day 3 or day 4, depending on the dose of responder cells; by day 6 the response often returns to near-background levels. Third, Lyt-2⁺ cells respond well to class I and full H-2 differences, but give little or no response to class II differences. Within the first 3–4 days of culture, MLR of Lyt-2⁺ cells, e.g., to a full H-2 difference, are generally *higher* than MLR of L3T4⁺ cells to this difference. It should be mentioned that the response of B6 T cells to bml is appreciably higher with purified Lyt-2⁺ cells than with unseparated T cells.

In addition to responding to bm1, B6 Lyt-2$^+$ cells respond well to other class I-different mutants, including bm4, bm9, bm10, and bm11. The amplitude of MLR to these mutants varies considerably, the highest responses being seen with bm1 and bm11 and the lowest responses with bm9. In the case of allelic class I differences, studies with the B10.A ($K^kI^kD^d$) strain has shown that purified Lyt-2$^+$ cells respond well to both H-2K (K^q, K^s) and H-2D (D^k, D^b) differences. As with the response to mutant class I differences, MLR directed to allelic class I differences are higher with purified Lyt-2$^+$ cells than with unseparated T cells.

Two trivial explanations could account for the MLR observed with Lyt-2$^+$ cells. First, it might be argued that the responding cells receive help in the form of IL-2 from L3T4$^+$ cells in the stimulator population. This possibility seems most unlikely because pretreatment of the stimulator population with anti-Thy-1 MAb + C′ does not diminish MLR (Table I). The second objection is that, despite the failure to detect L3T4$^+$ cells in the purified Lyt-2$^+$ cell population, there is nevertheless sufficient contamination with L3T4$^+$ cells to provide a minimum amount of help (IL-2) required by the Lyt-2$^+$ responders. According to this possibility, addition of anti-L3T4 MAb to the cultures would be expected to block MLR of Lyt-2$^+$ cells. This is not the case. Although anti-L3T4 MAb is highly effective at inhibiting MLR of B6 L3T4$^+$ cells to bm12, this antibody fails to block the response of B6 Lyt-2$^+$ cells to bm1. By contrast, anti-Lyt-2 MAb abolishes B6 Lyt-2$^+$ anti-bm1 MLR but does not affect B6 L3T4$^+$ anti-bm12 MLR.

Stimulator Cells for Mixed-Lymphocyte Reactions of Lyt-2$^+$ Cells

The capacity of Lyt-2$^+$ cells to produce MLR to a full H-2 difference does not require class II compatibility between responders and stimulators. Thus, the response of B6 Lyt-2$^+$ cells to the K^k and D^k alloantigens of CBA ($K^kI^kD^k$) is as high with homozygous stimulators as with heterozygous [(B6 × CBA)F_1] stimulators. This finding, together with the failure of Lyt-2$^+$ cells to respond to an isolated class II difference and the inability of anti-L3T4 MAb to block anti-class I MLR, strongly suggests that the response of Lyt-2$^+$ cells to class I differences does not involve co-recognition of class II molecules. Surprisingly, however, class II$^+$ (Ia$^+$) cells do seem to play an important role in controlling anti-class I MLR. Thus, it has been found that pretreatment of stimulator cells with anti-Ia MAb plus C′ virtually abolishes MLR of purified Lyt-2$^+$ cells. The significance of these findings will be discussed later. Some of the features of MLR mediated by Lyt-2$^+$ vs. L3T4$^+$ cells are summarized in Table II.

Table II. Features of Primary Mixed-lymphocyte Reactions Mediated by L3T4$^+$ vs. Lyt-2$^+$ Cells

	Responder cells	
Features of MLR	*L3T4$^+$*	*Lyt-2$^+$*
Stimulus for MLR	Allo class II determinants	Allo class I determinants
Auto MLR	High	Low
Kinetics	Late peak	Early peak
Inhibition with anti-L3T4 MAB	+	–
Inhibition with anti-Lyt-2 MAB	–	+
Requirement for Ia$^+$ stimulators	+	+
Inhibition with anti-Ia MAB	Strong	Detectable, but weak

Cell-mediated Lympholysis by Purified Lyt-2$^+$ Cells

Although information on CML responses mediated by purified Lyt-2$^+$ cells is still rather limited, it is clear that B6 Lyt-2$^+$ cells cultured for 4 days with irradiated, anti-Thy-1 + C′-treated bm1 stimulators mediate strong CML responses to bm1 targets. A typical experiment with ^{51}Cr-labeled concanavalin A (Con A) blasts as targets is illustrated in Table III. It can be seen that high levels of lysis occurred irrespective of whether CTL were generated from purified B6 Lyt-2$^+$ cells or from unseparated B6 T cells. Cross-reactive lysis on bm9 targets was low and there was no lysis of syngeneic targets.

In Vivo Responses of Purified T Cell Subsets

Several approaches have shown that, as in vitro, isolated populations of L3T4$^+$ and Lyt-2$^+$ cells function well in vivo. Proliferative responses of T cells to alloantigens in vivo can be assayed by injecting T cells into semiallogeneic (F_1) irradiated mice (12). Proliferation of the donor T cells in the spleen is measured by injecting the mice with tritiated thymidine ([^{3}H]TdR) intravenously, removing the spleen within 1 hour, dissolving the spleen in an organic solvent, and then counting the radioactivity in

a liquid scintillation counter. Radioactivity levels in the spleens of mice given syngeneic T cells serve as a control; background counts observed in spleens of mice given no cells are subtracted from the data.

The results of a typical in vivo MLR are shown in Table IV. In corroboration of the data obtained in vitro, it can be seen that B6 Lyt-2$^+$ cells proliferated extensively in irradiated (B6 × bm1)F_1 mice but not in (B6 × bm12)F_1 mice. Conversely, B6 L3T4$^+$ cells responded well in (B6 × bm12)F_1 mice but not in (B6 × bm1)F_1 mice.

After proliferating in the spleen, T cells responding to alloantigens enter thoracic duct lymph in large numbers as blast cells (13). The numbers of blast cells entering thoracic duct lymph closely reflect the degree of proliferation in the spleen. An experiment in which thoracic duct lymphocytes (TDL) were recovered from irradiated bm1 mice given B6 L3T4$^+$ or Lyt-2$^+$ cells 4 days before is shown in Table V. It can be seen that injection of 3 × 10^6 B6 Lyt-2$^+$ cells yielded >2 × 10^7 lymph-borne cells over a drainage period of 24 hours; the vast majority of these cells were blasts. Only 1–2 × 10^6 TDL were recovered from bm1 recipients of B6 L3T4$^+$ cells; most of these cells were small lymphocytes. Reciprocal results occur with transfer of B6 T cell subsets to irradiated bm12 mice, entry of blast cells into the lymph being high with injection of L3T4$^+$ cells but only very low with transfer of Lyt-2$^+$ cells.

Table III. Capacity of Purified B6 Lyt-2$^+$ Cells to Generate Primary Cell-mediated Lympholysis Responses against bm1 Stimulators

Cells cultured with irradiated bm1 stimulators	*Killer to target ratio*	*% specific ^{51}Cr release with target cells*		
		bm1	*bm9*	*B6*
B6 T cells (unseparated)	33:1	58.1	8.5	0
	4:1	33.7	1.2	0
B6 Lyt-2$^+$ cells	33:1	57.2	7.4	0
	4:1	31.1	0.6	0

Aliquots of 2 × 10^6 responder T cells and 5 × 10^6 1,500-rad stimulators were cultured in a volume of 2 ml for 4 days. After harvesting, the T cells were incubated for 3 hours with ^{51}Cr-labeled Con A blast cells.

Table IV. Proliferative Response of T Cell Subsets to Class I vs. Class II H-2 Differences In Vivo

T cells transferred to irradiated mice	*Time after T cell transfer*	*[³H]TdR incorporation in spleens of irradiated mice*	
		(B6 × bm1)F₁	*(B6 × bm12)F₁*
		cpm × 10⁻³	
(B6 × bml)F₁	Day 4	0	—
	Day 5	0	—
(B6 × bml2)F₁	Day 4	—	0
	Day 5		5(± 3)
B6 Lyt-2⁺	Day 4	239(+ 52)	10(± 18)
	Day 5	449(+ 65)	12(± 10)
B6 L3T4⁺	Day 4	12(+ 14)	334(± 42)
	Day 5	0	101(± 28)

Lymph node T cells (5×10^5) or T cell subsets were transferred intravenously into mice (three mice/group) given 950 rads 6 hours before. As described in detail elsewhere (12), groups of the recipients were injected intravenously with 25 μCi[³H]TdR at day 4 or day 5 after T cell transfer. Spleens were removed 30 minutes later and dissolved in an organic solvent (Solusol). After addition of scintillation fluid, the solutions were counted in a β-counter. Background counts observed in irradiated mice given no cells ($10–20 \times 10^3$ cpm) have been substracted from the data.

Discussion

The data summarized in this chapter indicate that purified populations of Lyt-2⁺ cells can exert strong MLR and CML responses to H-2 alloantigens in the apparent absence of help provided by Lyt-2⁻ cells. The possibility that T cells in the stimulator population provide exogenous help would seem to be ruled out by the finding that pretreatment of stimulators with anti-Thy-1 MAb + C′ fails to diminish the response. Likewise, help from L3T4⁺ cells contaminating the responder population is rendered unlikely by the failure of anti-L3T4 MAb to inhibit the response.

In light of the well-entrenched view that Lyt-2$^+$ cells fail to differentiate in the absence of help from Lyt-2$^-$ cells, the above data might seem surprising. It should be pointed out, however, that at least two other groups—one working with mice (14) and the other with rats (15)—have concluded that purified Lyt-2$^+$ cells (or the OX-8$^+$ counterpart in rats) do mount high primary MLR. Likewise, primary B6 anti-bm1 CTL responses are reported not to require help from Ia-restricted Lyt-2$^-$ cells (16). One may also cite evidence that certain Lyt-2$^+$ T cell clones proliferate in vitro in the absence of exogenous help (17, 18), and that purified Lyt-2$^+$ cells can elicit lethal graft-versus-host (GVH) disease directed to minor histocompatibility antigens (19).

How then does one account for the dogma that MLR are directed predominantly to class II rather than class I differences? Several points can be made here. First, as mentioned earlier some workers do find appreciable MLR to class I differences (7–9). The fact that such responses tend to be lower than to class II differences might, at least in part, be a reflection of a low precursor frequency, Lyt-2$^+$ cells usually being outnumbered by L3T4$^+$ cells by a ratio of 2–3:1. In our hands, enrichment for Lyt-2$^+$ cells causes a corresponding elevation of MLR. Another point worth mentioning is that, at least for B6 responder cells, MLR by Lyt-2$^+$ cells tend to reach a peak on day 3 or 4 and are quite low thereafter. Measuring responses only on day 5 or 6 can thus be deceptive. The third

Table V. Numbers of Thoracic Duct Lymphocytes Collected from 950-rad (B6 × bm1)F_1 Mice Given B6 L3T4$^+$ or Lyt-2$^+$ Cells 4 Days Previously

Cells transferred to irradiated (B6 × bm1)F_1 mice	*Number of TDL collected over 24 hours*	*% Staining with antibodies specific for*		
		Thy-1	*L3T4*	*Lyt-2*
–	0.7×10^6	92	64	14
3×10^6 B6 L3T4$^+$	1.6×10^6	98	92	4
3×10^6 B6 Lyt-2$^+$	21.4×10^6	99	1	99

T cells were injected intravenously 6 hours after irradiation. Mice were cannulated 4 days later and TDL were collected over 24 hours. Aliquots of cells were incubated with anti-Thy-1, anti-L3T4 or anti-Lyt-2 MAB (all of rat origin) followed by a fluorescein-labeled mouse anti-rat Ig MAB. Cells were analyzed with a fluorescence-activated cell sorter.

point to emphasize is that although purified Lyt-2$^+$ cells invariably give highly significant MLR in our hands, most of the data have been obtained with T cells from mice of the B6 or B10 background. Studies with other strains are still rather limited. It should also be emphasized that the amplitude of MLR observed with Lyt-2$^+$ cells varies considerably, some mutant and allelic class I differences giving only quite low responses. Such variability might simply reflect a difference in precursor frequency for the determinants in question. Alternatively, unprimed Lyt-2$^+$ cells might consist of a mixture of helper-dependent (HD) and helper-independent (HI) cells, only HI cells being able to evoke MLR. The ratio of HI to HD cells might vary according to the particular class I difference involved, differences eliciting a weak MLR reflecting a high proportion of HD cells.

In contrast to the studies of other workers who used unseparated T cells (8, 9), we have observed no evidence that anti-class I MLR mediated by purified Lyt-2$^+$ cells involves recognition of class II molecules, at least in the case of B6 anti-bm1 MLR. It is clear nevertheless that removal of Ia$^+$ cells from the stimulator population abolishes the response (see also 8, 9). The simplest explanation for this finding is that, in addition to recognition of antigen, the induction of Lyt-2$^+$ cells requires recognition of a "second signal" provided by the antigen-presenting cells (20). Delivery of this second signal might be a property unique to Ia$^+$ cells, but not necessarily involve recognition of Ia molecules per se. In an attempt to define which particular cell types can convey a putative second signal, we are currently testing a variety of tumor cells for their capacity to stimulate MLR by Lyt-2$^+$ cells. Interestingly, the P815 mastocytoma—which is Ia$^-$—has proved to be a good stimulator for Lyt-2$^+$ cells, though not for L3T4$^+$ cells. Thus the capacity to stimulate Lyt-2$^+$ cells might not be unique to Ia$^+$ cells.

In addition to responding well in vitro, Lyt-2$^+$ cells also give strong proliferative responses to class I differences in irradiated mice. As in vitro, Lyt-2$^+$ cells respond in vivo only to class I and not class II differences. Conversely, L3T4$^+$ cells respond only to class II and not class I differences, both in vivo and in vitro. These findings provide support for the suggestion of others (3) that L3T4 and Lyt-2 molecules guide T cell specificity for class II and class I molecules, respectively, perhaps by binding to invariant epitopes expressed on these H-2 molecules.

Very recent studies have shown that, in addition to responding to H-2 alloantigens in vivo in terms of proliferation, L3T4$^+$ cells and Lyt-2$^+$ cells also mediate GVH disease ([21] and unpublished data of the author). Two types of GVH disease have been studied, i.e., lethal GVH disease in irradiated mice and splenomegaly in neonatal mice. As for MLR, highly

purified Lyt-2$^+$ cells produce GVH disease directed selectively to class I determinants whereas L3T4$^+$ cells account for anti-class II GVH disease. In the case of splenomegaly in neonates (measured at day 9 after transfer), we have seen no evidence that the function of Lyt-2$^+$ cells requires interaction with L3T4$^+$ cells. Interestingly, however, some form of T-T interaction might be required for lethal GVH disease (22). Thus, although B6 Lyt-2$^+$ cells alone cause 100% mortality in irradiated (B6 × bm1)F_1 mice, addition of L3T4$^+$ cells significantly shortens the mean survival time. With transfer of Lyt-2$^+$ cells alone, the recipients become ill during the second week after transfer but then appear to recover. At about 4 weeks after transfer, however, the mice deteriorate quite suddenly and die within a few days.

Our working hypothesis for these findings is that Lyt-2$^+$ cells function in a HI fashion within the first week or so but then become dependent on exogenous help. If help is provided in the form of injected L3T4$^+$ cells, the Lyt-2$^+$ cells continue to respond to the host alloantigens and the mice die from acute GVH disease. Without exogenous help the Lyt-2$^+$ cells enter a quiescent phase, but then become reactivated when newly formed L3T4$^+$ cells leave the host thymus in the third and fourth weeks after irradiation. These cells release IL-2 upon contact with environmental antigens ("bystander help"). According to this idea, thymectomized recipients of purified Lyt-2$^+$ cells would not be expected to show a recrudescence of GVH disease at 4 weeks after transfer. Experiments are underway to test this prediction.

Summary

Until recently, it has been generally held that the differentiation of Lyt-2$^+$ precursor cells to CTL depends heavily on the availability of "help" (interleukin 2) from the L3T4$^+$ subset of T cells. This notion is not supported by evidence summarized in this chapter, which suggests that purified Lyt-2$^+$ cells function effectively in the apparent absence of help provided by other cells. Purified Lyt-2$^+$ cells give high primary MLR and CTL responses to H-2 alloantigens, both in vitro and in vivo, the specificity of the response being directed selectively to class I and not class II H-2 differences. Whereas anti-Lyt-2 MAb ablates the response of Lyt-2$^+$ cells in culture, no inhibition is seen with anti-L3T4 MAb. Purified L3T4$^+$ cells also give strong MLR to H-2 differences, but these cells respond only to class II and not class I differences; only anti-L3T4 and not anti-Lyt-2 MAb inhibits MLR of L3T4$^+$ cells. Two conclusions follow from these findings. First, the data support the view that selective

recognition of class I vs. class II H-2 molecules is guided by cell surface Lyt-2 and L3T4 molecules, respectively. Second, the notion that the activation of unprimed Lyt-2$^+$ cells depends critically on help provided by L3T4$^+$ cells will need to be revised.

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Linked Recognition for T-T Collaboration In Vivo

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The immune system has often been described as a two-edged sword. It protects against disease. This is of obvious benefit. However, it can respond against self antigens, resulting in autoimmunity. In addition, the immune response against transplanted foreign tissue continues to be a major concern of surgeons and immunologists. Because thymus-derived T lymphocytes mediate the rejection process (1), considerable interest has been focused on how T cells function. Certain assumptions about T cell-mediated graft rejection in vivo have relied heavily on conclusions from studies performed in vitro. This chapter discusses our studies on T cell activation in vivo. Our conclusions point to differences between T cell activation in vitro and T cell activation in vivo.

There are at least two signals involved in T cell activation (2, 3). One signal occurs as antigen is bound by the T cell receptor(s). Antigen is normally presented to T cells by specialized antigen-presenting cells (APC), usually described as macrophage-like in morphology and gamma-radiation resistant (4, 5). That APC present the antigen is important for at least two reasons. First, the vast majority of T cells only respond to antigen when it is presented in conjunction with either class I (murine K,D-like) or class II (murine Ia) molecules coded for by the major histocompatibility complex (MHC) (6, 7). Antigen-presenting cells, bearing MHC antigens, present soluble or particulate antigen in an MHC-restricted fashion to T cells. The evidence suggests that, in addition to presenting antigen, APC also provide an additional signal in the form of the lymphokine interleukin 1 (IL-1) (8, 9). These two signals, antigen in association with MHC molecules and IL-1, initiate T cell activation. After activation, T cells produce interleukin 2 (IL-2) (10). Interleukin 2 is a T cell growth factor that promotes clonal expansion (9–11).

T cells collaborate with B cells in the production of antibody. This is accomplished, in part, via the production by helper T cells (Th) of interleukins required for B cell differentiation (12). The experiments of Cantor and Boyse (13) demonstrated that Th bore distinct cell surface

antigens with which they could be distinguished from another subset of T cells, termed cytotoxic T lymphocytes (CTL). Cytotoxic T lymphocytes can directly lyse other cells. These authors presented evidence that Th also provided help for the generation of CTL (14). Other studies reported that Th also produce helper factors for T cell growth and differentiation (11). These observations provided experimental support for models of T-T collaboration (15–17). Simply stated, it was proposed that T cells could be divided into two subsets, Th and CTL precursors (CTL-p). The majority of Th were activated by recognition of class II-associated antigen presented by APC. Once activated, these Th produce IL-2 required for the IL-2-dependent CTL-p to become CTL. A simplified way to state this is that the second signal for CTL-p is not IL-1.

We have been studying whether T-T collaboration, as proposed above from in vitro studies, applies in vivo. In particular, we have attempted to discriminate among three possible models. In the first model (Figure 1), CTL-p, unlike Th, need not be in contact with APC in order to become activated. This model is derived from the observation by several groups of investigators (18–20) that the requirement for the APC in the generation of CTL can be circumvented in vitro by the addition of IL-2-containing supernatants from mitogen-activated T cells. In the second model, the CTL-p require direct contact with APC in vivo to become activated. This allows the CTL-p to be in proximity to their source of IL-2, provided by the interaction between APC and Th. In the third model, CTL-p, like Th, are activated by the same basic, two-signal pathway of antigen and IL-1. This leads to IL-2 production by both populations (21).

The approach that we have employed in vivo to discriminate among these models is termed negative selection. Negative selection centers on the observations made first by Gowans (22) that T lymphocytes recirculate from blood to lymph and back again. Therefore, if one injects lymphocytes into the blood, they can be collected as soon as 6 hours later in the thoracic duct lymph, a primary efferent lymphatic returning lymphocytes to the blood. The observation of particular interest to our work is that recirculating lymphocytes are removed from this recirculating pool when they confront antigen (23). Therefore, if one injects cell-bound lymphoid alloantigens of strain B, for example, into the blood stream of an allogeneic and MHC-incompatible strain A recipient and collects the thoracic duct lymphocytes (TDL) from the injected animal (Figure 2), these TDL show a diminished response to B strain stimulator populations as assessed by in vivo or in vitro assays. This period of reduced responsiveness of the TDL to injected antigen is termed the period of negative selection (A negative to B). The sequestered cells divide and

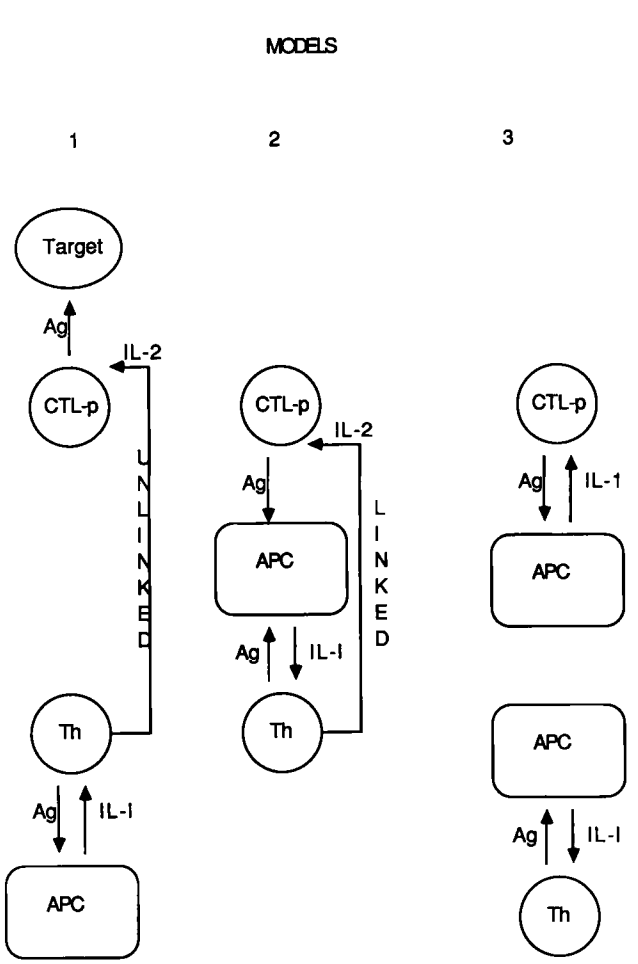

Figure 1. Possible models of T-T collaboration.

their progeny are released into the recirculating pool in such a manner that one observes an enhanced response, the period termed positive selection (24). Negative selection was demonstrated as the absence of proliferation (25) and the failure to generate CTL against B but not C stimulator cells in vitro (25–27) in the TDL of strain A animals injected with strain B alloantigen. From these observations, we concluded that negative selection after the injection of B strain lymphoid cells resulted in a specific depletion of both Th (proliferating) and CTL-p (cytotoxic) subsets (25, 26).

Negative selection, demonstrated as a withdrawal of T cells from the recirculating pool, simply could reflect antigen binding by the responding

T cells. If this were the case, the selection of CTL-p would require only one signal, the recognition of antigen. Alternatively, selection of CTL-p might reflect T cell activation and thus require two signals. To test this we injected A strain animals with B strain tumor cells, rather than B strain lymphoid cells. The tumor population differed from the lymphoid population in that it failed to stimulate purified A-strain T cells to generate CTL responses in vitro in the absence of APC or exogenous sources of IL-2-containing supernatants (26). This deficiency for stimulation in vitro likely results from the failure of the tumor to produce an interleukin-mediated second signal. The B tumor also differed in its capacity to induce negative selection. Unlike the B lymphoid population, which induced negative selection of Th and CTL-p, the B tumor only induced negative selection of Th. Further study showed that these Th were probably specific for tumor antigens presented by the strain A APC. Negative selection of CTL-p did occur if the animals were perfused with IL-2-containing supernatants during the selection period after tumor injection. These results are summarized in Table I. Therefore we concluded (26) that CTL-p required two signals for negative selection to occur. Furthermore, we concluded that the two signals must be delivered simultaneously. This was inferred from the fact that activation by host APC of Th was not sufficient to provide the second signal for tumor specific CTL-p. This is presumably because the Th and CTL-p are specific for tumor antigens presented by different cells, Th by APC and CTL-p by the intact tumor.

In contrast to tumor cells, the injection of allogeneic lymphoid cells did select CTL-p in the absence of IL-2-containing supernatants (25, 26). The simplest explanation for this is that APC in this lymphoid population interact with both Th and CTL-p resulting in the exposure of the CTL-p to a high local concentration of IL-2 (model 2). Alternatively,

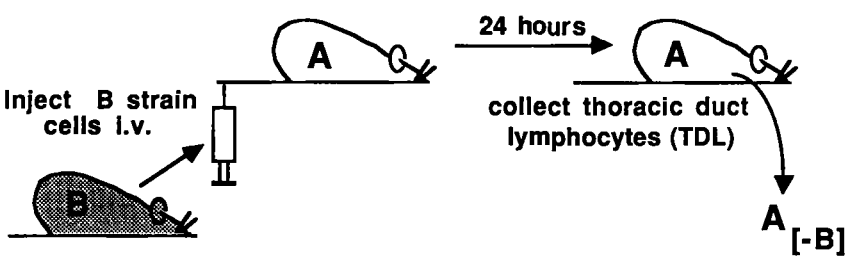

Figure 2. Negative selection of antigen-specific T cells in vivo.

Table I. Negative Selection after the Intravenous Injection of Various Allogeneic Populations

		Negative selection of T cell reactive to			
		B		*B tumor*	
Experiment	*T cells tested*	*Th*	*CTL-p*	*Th*	*CTL-p*
1	A	No	No	No	No
2	A–B	Yes	Yes	No	No
3	A–B tumor	No	No	Yes	No
4	A–B Tumor + ConASnF	NT	Yes	Yes	Yes

Thoracic duct lymphocytes were collected from A strain rats. In experiments 2–4, the TDL were from animals injected i.v. at least 24 hours previously with (experiment 2) more than 400 million allogeneic and MHC-incompatible strain B lymph node and spleen cells, (experiment 3) more than 30 million tumor cells from an adenovirus 2 transformed embryo fibroblast line from strain B, or (experiment 4) the strain B tumor cells followed immediately by a 24-hour i.v. infusion of supernatant factor from Concanavalin A-activated spleen cells (ConASnF) devoid of ConA by passage through a G100 column. The total amount of ConASnF approximated three spleen donor equivalents. Experiment 1 is a control: TDL from uninjected animals. In all four experiments the TDL were incubated on nylon wool columns and the nonadherent population, highly enriched for T cells and depleted of Ia and immunoglobulin-bearing cells, was cultured with gamma-irradiated strain B lymph node cells or B strain tumor in vitro. After 6 days the cultures were harvested and tested for the presence of Th and CTL specific for strain B lymphoid or tumor cells. Negative selection of Th was defined as the inability of responding T cells to generate cytotoxic activity when stimulated in vitro with gamma-irradiated strain B lymphoid or tumor cells in the absence of exogenous sources of ConASnF. Negative selection of CTL-p was defined as the absence of CTL in cultures stimulated with B lymphoid or B tumor cells even when the culture medium was supplemented with ConASnF.

NT, not tested.

the APC might release factors such as IL-1 which, in the absence of Th, facilitate activation of CTL-p (model 3). The next series of experiments was designed to distinguish between models 2 and 3.

Gamma- and ultraviolet (UV)-irradiated lymphoid populations differ in their capacity to stimulate T cells in vitro (25). Gamma-irradiated populations stimulate proliferative and cytotoxic responses in vitro whereas UV-treated populations do not. This seems not to be due to a reduction

in antigen after UV treatment because CTL responses do occur if IL-2-containing supernatants are cocultured with UV alloantigen. The generally accepted explanation for the lack of stimulation by UV alloantigen is that the APC are killed by UV irradiation and can no longer produce IL-1 (28), the product of a metabolically active cell. Antigen-presenting cells are resistant to gamma-irradiation and therefore continue to be effective at stimulating in vitro. When UV- and gamma-irradiated populations from strain B were injected into strain A to measure their capacity to induce negative selection, both gamma- and UV-treated populations induced negative selection as assessed by absence of proliferation to B stimulator cells in vitro. Cytotoxic T lymphocyte responses were also negatively selected. However, selection with UV-irradiated alloantigen differed

Table II. A Comparison of Negative Selection with Gamma- and UV-irradiated Lymphoid Populations

Capacity to allogeneic population to induce	*In vitro assay used*	*Treatment*	
		Gamma	*UV*
Activation in vitro	^{3}H	Yes	No
	CTL	Yes	No
	^{3}H + ConA SnF	Yes	No
	CTL + ConA SnF	Yes	Yes
Negative selection in vivo	^{3}H	Yes	Yes
	CTL	Yes	Yes
	^{3}H + ConA SnF	Yes	Yes
	CTL + ConA SnF	Yes	No
Positive selection in vivo	^{3}H	Yes	No
	CTL	Yes	No

Thoracic duct lymphocytes from A strain animals, injected with gamma- or UV-irradiated strain B lymphoid cells, were tested for evidence of negative or positive selection. Negative selection was defined as the absence of proliferative ([^{3}H] thymidine incorporation) or cytotoxic T cell responses (the ability to lyse chromium labeled target cells syngeneic to the cells used to stimulate) to gamma-irradiated stimulators in vitro in the presence or absence of supernatant factor from concanavalin A-activated spleen cells (ConASnF). Positive selection was defined as an enhanced response compared to TDL from uninjected animals.

^{3}H, thymidine incorporation.

in two respects (25). First, CTL responses were restored when IL-2-containing supernatants were added. Secondly, there was no evidence of positive selection in the TDL of UV alloantigen-injected animals; i.e., the TDL collected 5 days after antigen injection did not respond in an enhanced fashion compared to TDL from uninjected animals. Thoracic duct lymphocytes from gamma-alloantigen-primed animals responded in an enhanced fashion. These results are summarized in Table II.

These experiments show that the capacity of the CTL-p population to be negatively selected is linked to the capacity of another T cell subset to proliferate (the Th negatively selected by UV alloantigen). Because the CTL-p population seems to require IL-2, failure of the Th to proliferate and produce IL-2 could explain why UV-irradiated alloantigen does not induce negative selection of CTL-p. These data and their interpretation do not support model 3 where CTL-p require close contact with an APC in order to receive IL-1 only. Instead, we conclude that CTL-p need to be in contact with APC to be in the proximity of Th cells which provide IL-2 (model 2).

In summary, activation of the majority of CTL-p in vitro in most cases requires the cooperation of helper T cells. The Th cells require APC for activation to occur. Once the Th–APC interaction has taken place, various soluble factors are released and these are sufficient to induce the transition of the CTL-p to CTL. This conventional picture cannot be transferred directly to happenings in vivo. Here Th cells may be activated in the absence of CTL-p activation. The difference appears to rest on the absence either in the right spot or the right concentration of the soluble factors required for the CTL-p to CTL transition. A caveat in these in vivo studies is that we sometimes have to equate removal from the recirculating pool with activation. However, the difference between the effect of tumor cells and gamma- and UV-irradiated lymphoid cells points to the need for intimate contact among CTL-p, Th, and APC. This requirement apparently can be circumvented in vitro by confining Th and CTL-p, specific for antigens on different APC, within a tissue culture vessel. Under more rigorous compartmentalization in vivo it would be unlikely that these two T cells would ever be close enough for an effective collaborative interaction to occur.

The studies reviewed in this text provide further support for the concept that, like T-B, T-T collaboration involves linked recognition of antigen. For effective CTL generation in vivo, the CTL target and Th antigen must be presented by the same APC in order to transmit the helper signal efficiently.

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Phagocytes and Killer Cells

Trophic and Defense Functions of Murine Macrophages

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Introduction

Macrophages (Mϕ) play an important role in phagocytosis and host defense against infection, in collaboration with antibody, complement, and T lymphocytes. The Mϕ contribute as effector cells to both humoral and cell-mediated immunity, but their relationship to dendritic cells and function in induction of the immune response remains controversial. Three types of Mϕ can be distinguished in peripheral tissues of the mouse, namely, resident, elicited (e.g., by a sterile inflammatory stimulus), or immunologically activated, in which lymphokines, especially interferon-gamma, induce an enhanced Mϕ ability to kill various microbial and cellular targets. Although the cytocidal activities of elicited and activated Mϕ vary greatly, these populations share the property of induced recruitment from blood by an inflammatory or infectious agent, unlike resident cells in which the nature of the stimulus controlling constitutive emigration into tissues is unknown.

Resident and recruited Mϕ populations isolated from the peritoneal cavity of the mouse have been studied intensively, but very little is known about the properties of Mϕ elsewhere. Earlier investigators appreciated the widespread distribution of cells of the "reticulo-endothelial system," their role in clearance of injected particles such as carbon from the circulation, and their contribution to innate and acquired immunity. More recently, Mϕ have been localized in many tissues with the aid of monoclonal antibodies (MAbs) and some of these cell populations have

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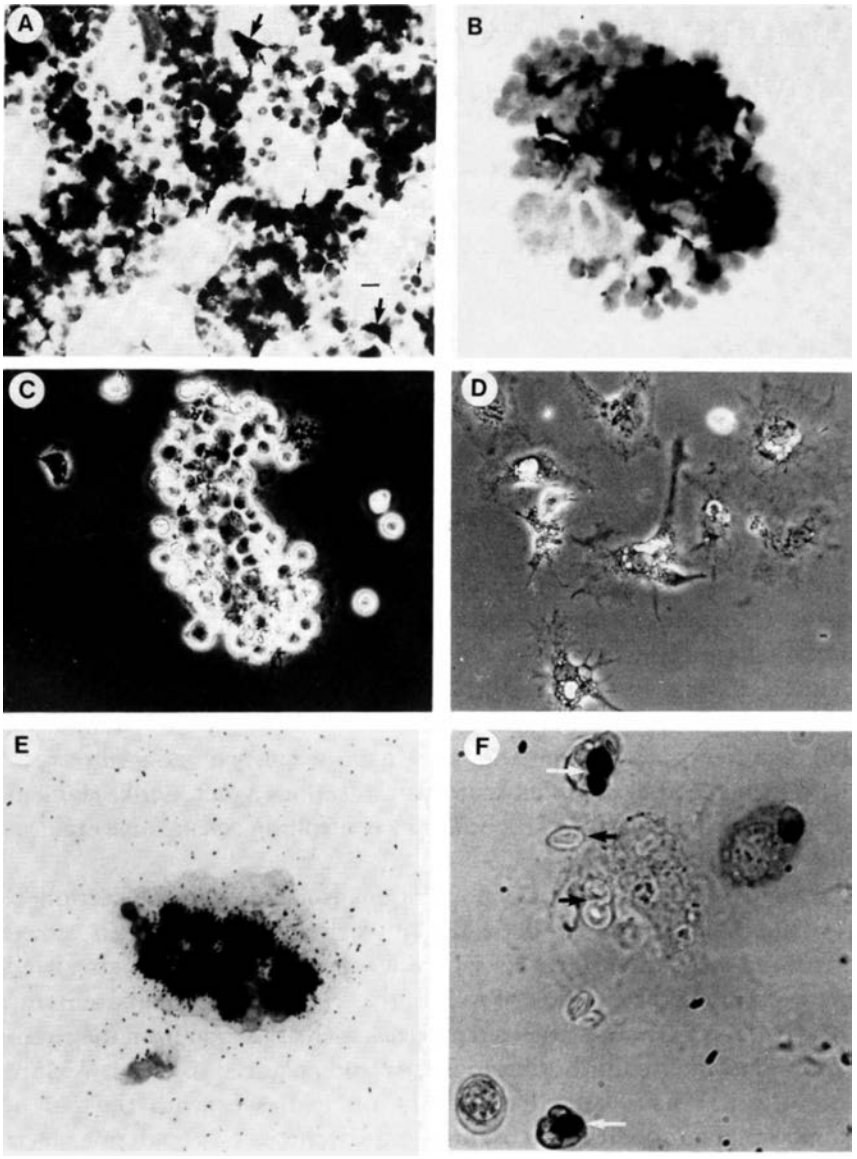

Figure 1. Stromal Mϕ in mouse bone marrow before and after isolation. An example of a resident Mϕ population that performs trophic rather than cytocidal defense functions. (A) In situ. F4/80+ Mϕ with extended plasma membrane processes in hemopoietic islands. (B) After collagenase digestion. Hemopoietic cluster isolated by sedimentation through a fetal bovine serum column shows F4/80+ Mϕ and F4/80− erythroid and myeloid cells. (C and D) After adherence to glass. (C) Cluster on surface of Mϕ with extended plasma membrane processes. (D) Macrophages stripped free of associated cells. (E) Autoradiographic demonstration that hemopoietic cells in a cluster synthesize DNA (incorporate

been isolated from organs such as liver, spleen, bone marrow, and skin. Resident tissue Mϕ display interesting differences from resident peritoneal cells and from recruited cells at sites of inflammation and infection, indicating that these Mϕ may contribute different functions to the host. Newly recruited monocytes and elicited/activated Mϕ display vigorous respiratory burst activity, which accounts for much of the augmented host response to infection, unlike resident peritoneal or tissue Mϕ that lack this activity. Resident Mϕ within tissues are closely associated with many different cells and extracellular matrix components and are well placed to perform a variety of trophic, rather than only phagocytic or destructive functions. These interactions with neighboring cells and their products include support of cell growth and differentiation and modulation of various metabolic activities. In this review, we compare properties of resident and recruited Mϕ populations in the mouse and contrast their mobilization and possible functions. Experimental details can be found in the references cited.

Distribution of Macrophages in the Normal Adult Mouse

The rat MAb F4/80 isolated by Austyn and Gordon in our laboratory (1) has been used to localize cells that express this antigen in various tissues of the mouse. The antibody (Ab) defines an epitope on a 160,000-mol wt plasma membrane glycoprotein of unknown function expressed by virtually all mature mouse Mϕ studied in situ or in vitro (for review, see reference 2). The antigen (Ag) is resistant to glutaraldehyde fixaton and it has been possible for Hume and Gordon (3) to detect its presence in perfusion-fixed, paraffin-embedded sections with excellent preservation of morphologic detail. The evidence for specificity of the reagent is discussed elsewhere (4). In selected tissues such as the central nervous system, the distribution of F4/80 Ag has been correlated (5) with that of other Ag found on mouse Mϕ, Mac-1 (CR3), and 2.4G2 (FcR IgG1/2b). The F4/80 Ab falls short of being a "pan-" Mϕ marker in that it fails to bind to progenitors of the Mϕ lineage (6), unlike the CSF-1 receptor, a distinct Mϕ-specific glycoprotein of approximately 160,000 mol wt (Crocker, P., and R. Stanley, unpublished observation) and it may not react with phagocytic Mϕ-like cells in spleen marginal zone (7).

[^{3}H]thymidine) on surface of a Mϕ. (F) Mature stromal Mϕ ingest zymosan (*black arrows*), but do not display a respiratory burst (as monitored by NBT reduction in which a dark blue formazan precipitate is deposited on the zymosan particle) unlike neutrophils and monocytes (*white arrows*). Adapted from References 12 and 16, which contain further details.

Although Ag F4/80 levels are decreased in activated Mϕ (8), it is still readily detected on these cells by immunocytochemistry (9).

The details of distribution of Ag F4/80 in normal adult murine tissue are described elsewhere (3, 4). F4/80+ cells are found in hemopoietic (Figure 1), lymphoid, and many other tissues, often at portals of entry, and are characteristically associated with endothelium, small blood vessels, and epithelium (Figure 2). The pattern of cell distribution implies a defined route of migration from blood into tissues and specific mechanisms of localisation. Studies by Lee et al. (10), in which a quantitative absorption assay with homogenized tissue was used, have confirmed the presence of relatively large amounts of F4/80 Ag in bone marrow, liver, and spleen, as well as in the gastrointestinal and genitourinary tracts.

Ontogeny of F4/80 + Cells

Unpublished studies by Morris, Shia, and Hume have shown that Ag F4/80 provides an excellent marker to detect Mϕ within the mouse embryo. F4/80+ cells appear first in yolk sac by day 9 and soon elsewhere at sites of hemopoietic activity and in tissues of mesodermal origin. Intense staining has been observed in fetal liver (after day 12) and subsequently in spleen and bone marrow where Mϕ with stellate processes are initially associated with clusters of nascent erythroid cells and later with myeloid cells. By contrast, yolk sac F4/80+ cells appear after, and are not obviously associated with, erythroid cells. The possible role of Mϕ in the hemopoietic microenvironment will be considered further below; in addition, these studies imply that there are distinct waves of migration of precursor cells and/or mature Mϕ during development, culminating in the adult distribution. Recruitment of monocytes during morphogenesis in the central nervous system was studied by Hume et al. (11) and Perry et al. (5). Senescent neurons and/or axons are phagocytosed by blood-derived monocytes, which subsequently differentiate into mature microglia. In the plexiform layers of the retina these Mϕ adopt a highly regular mosaic pattern of distribution resembling that of Langerhans cells in the basal layer of adult mouse epidermis (Figure 2) (12).

Recruitment of F4/80 + Cells during Inflammation and Infection, Discrimination from Resident Macrophages In Situ

We have examined several models of inflammation and infection. Large numbers of F4/80+ cells can be recruited to local sites such as the peritoneal cavity, liver, and spleen. The extent, duration, and site of accumu-

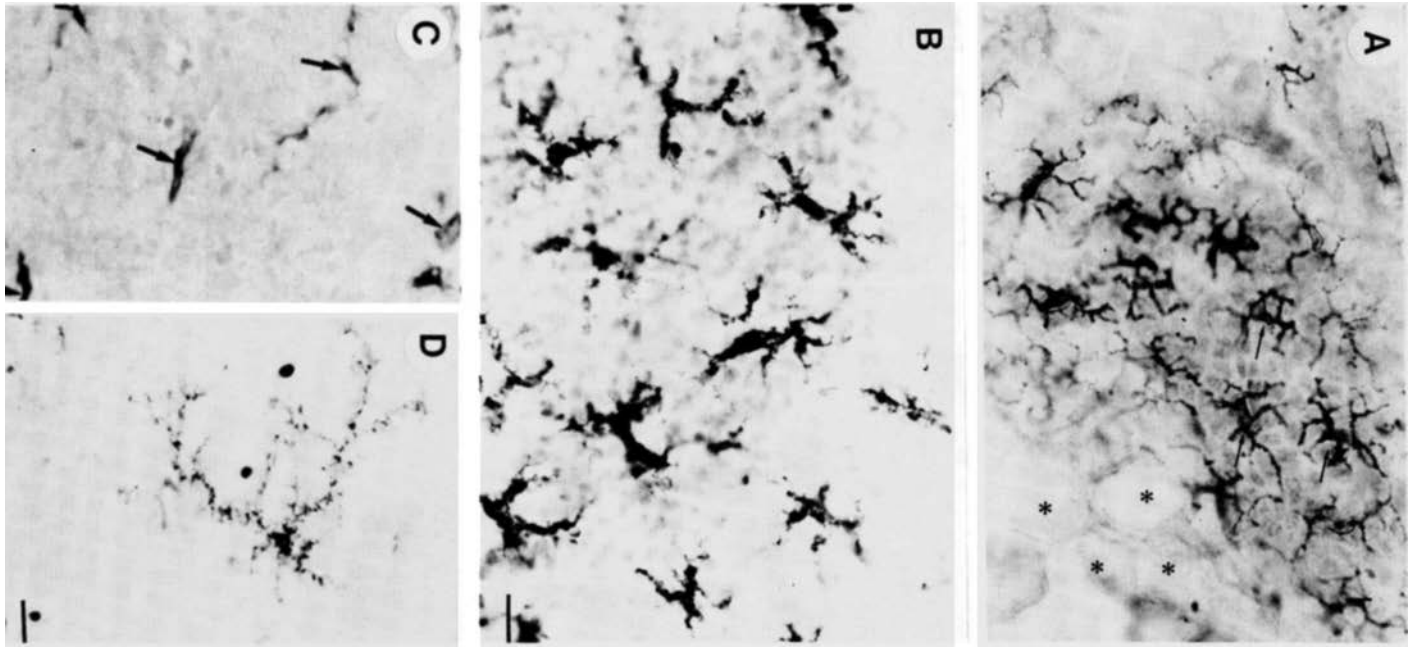

Figure 2. The morphologic similarity between F4/80+ Mϕ in epidermis (A: Langerhans cells), retina (B: inner plexiform layer) and in other complex epithelia (C: trachea). (D) Further elaboration of F4/80+ processes in microglia, after maturation in central nervous system. Note interactions with keratinocytes in panel A. Taken from References 11, 12, and 29.

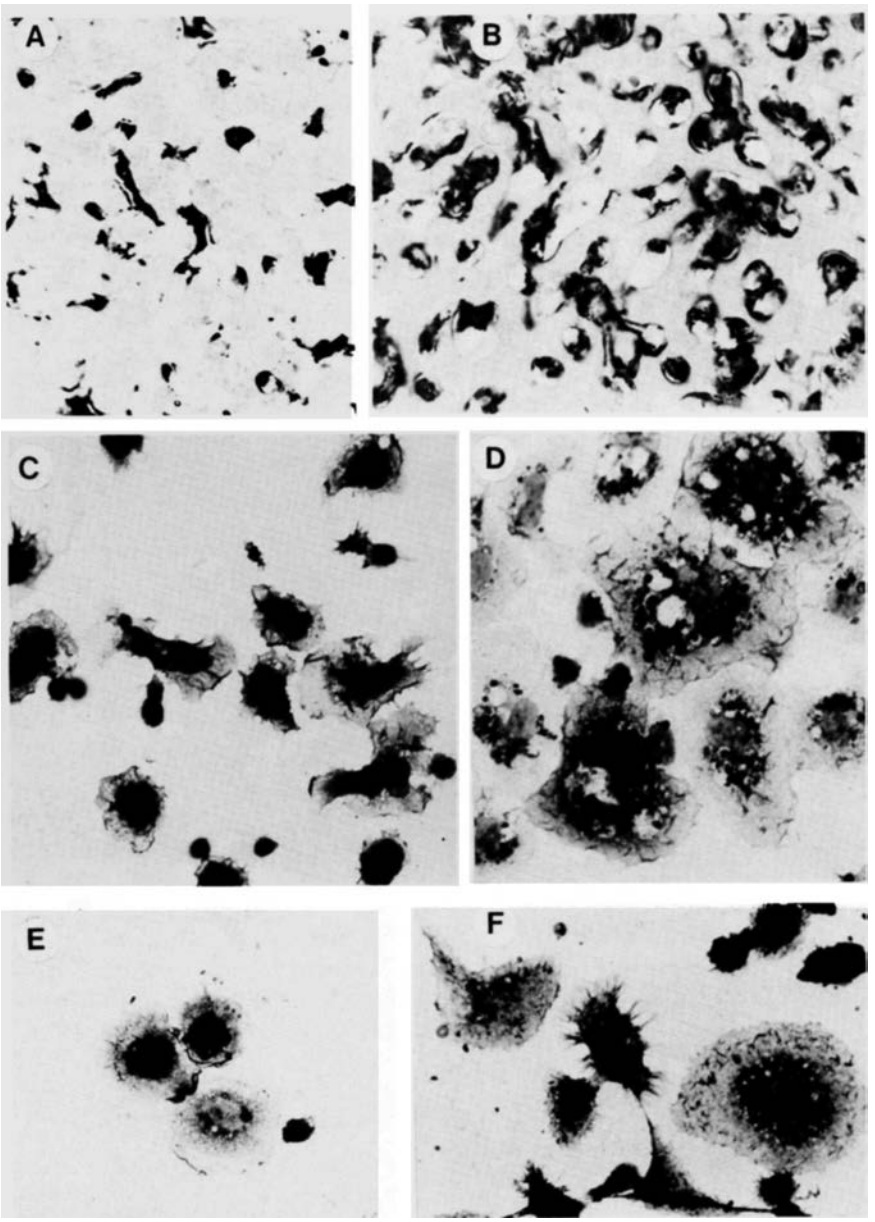

Figure 3. Effect of *P. yoelii* infection on Mϕ in liver (A–D) and blood (E, F), defined by F4/80 immunocytochemistry. (A and B) F4/80+ cells in liver of normal (A) and infected (B) mice. Macrophages remain sinus-lining although cell number and plasma membrane extent show a considerable increase. (C and D). Isolated liver Mϕ after collagenase digestion and adherence to glass. After infection (D), liver Mϕ are enlarged, spread rapidly, and contain pigment and erythrocyte debris, compared with normal Kupffer cells (C).

lation depend on the inducing agent. Administration of thioglycollate broth in the peritoneal cavity elicits a local influx of Mϕ which express low levels of F4/80 Ag (8), a reflection of their immaturity. Injection of *Corynebacterium parvum* or Calmette-Guerin bacillus (BCG) via the same route evokes a peritoneal exudate and striking focal accumulation of F4/80+ cells in granulomata in liver and elsewhere (Rabinowitz, S., unpublished observation). Unlike Mϕ elicited by thioglycollate broth or endotoxin, those induced by *C. parvum* or BCG express Ia and 7/4 Ag (a myelomonocytic differentiation Ag constitutively expressed by mouse neutrophils, but not resident peritoneal or tissue Mϕ [13]) and other endocytic and secretory changes associated with immune activation (8, Hirsch, S., unpublished observation). When the activating agent is blood-stage *Plasmodium yoelii* 17X, F4/80+ Mϕ accumulate in large numbers in blood, liver (Figure 3), and spleen (14). In liver, malaria-induced Mϕ accumulate diffusely throughout the portal bed rather than focally. Immunocytochemical studies show that newly recruited Mϕ, which enter the liver after *P. yoelii* infection, lose 7/4 and Mac-1 Ag, but retain Ag F4/80 as they adhere and spread on the sinusoidal wall. The presence of Ag 7/4 and Mac-1 distinguishes malaria-induced liver Mϕ from resident Kupffer cells that lack both markers, and these populations differ markedly in antimicrobial ability, as discussed below. This distinction between resident and recruited Mϕ leaves open the possibility of some local production of Mϕ within the inflamed liver. In normal uninfected spleen, which contains mature and newly generated cells as a result of local hemopoiesis (15), the phenotype of resident cells is more heterogeneous than in normal liver and it has not proved possible to distinguish malaria recruited and resident cells by Ag marker analysis (14). Local production of Mϕ is even more marked in the bone marrow, but mature as well as immature Mϕ populations can be distinguished here normally (12, 16) and after infection (14). After thioglycollate broth injection, local proliferation of Mϕ could occur to a limited extent in other peripheral sites such as the peritoneal cavity, although this is likely to contribute only a fraction of the 10-fold increase in cell numbers observed within a week.

After infection (F) F4/80+ cells in blood show very similar properties to those isolated from liver and spleen after infection, unlike normal monocytes (E). Mϕ isolated after malaria infection show features of immune activation (enhanced respiratory burst, decreased MFR). See Reference 14 for further details.

Isolation and Characterization of Macrophages from Different Sites

Some of the properties of resident, elicited, and activated peritoneal Mϕ are compared in Table I. There is little information on the properties of murine Mϕ isolated from other serous cavities, e.g., the pleura, or from internal compartments, e.g., the bronchoalveolar space, although this has been exploited as a readily available source of cells in other species (man, rabbit, guinea pig, rat). Because of low yield, murine monocytes have also been little studied. It is therefore not yet possible to draw firm conclusions as to regional differentiation of monocytes entering different sites within a single animal species in the normal, steady state or after introduction of an exogenous Mϕ-recruiting agent.

Table I. Characteristics of Freshly Isolated Resident, Elicited, and Immunologically Activated Mouse Peritoneal Macrophages

Property	*Resident*	*Elicited*	*Activated*
Growth (CSF-dependent)	0	+	+
Spreading	0	Enhanced	Enhanced
Ag F4/80	+	Reduced	Reduced
Ia	0	+	+ +
7/4	0	0	+
FcR	IgG1/2b	IgG1/2b and IgG2a	IgG2a Enhanced
CR3/Mac-1	+	+	+
MFR	+	+ +	Reduced
5′ Nucleotidase	+	Reduced	Reduced
Plasminogen activator	0	+	+
Respiratory burst (PMA)			
O_2^-	0	+	+
H_2O_2	0	0	Enhanced
Prostaglandin release	+ +	Reduced	Reduced
Apolipoprotein E release	+ +	Reduced	Reduced

Based on results obtained with thioglycollate-elicited and BCG-activated MO. These findings are broadly in agreement with results obtained with other eliciting and activating stimuli, where known. See References 25 and 36.

Even less is known about the properties of murine Mϕ embedded in solid organs and tissues. Such Mϕ often bear delicate and extensive plasma membrane processes that render their isolation difficult. Proteolytic digestion has been used to isolate Mϕ from bone marrow (16), liver (Kupffer cells) (14, 17), spleen (14, 18), lung (19), thymus (20), and skin (21). Unusual features have begun to emerge with regard to particular resident tissue Mϕ populations and will be compared with the properties of cells isolated from some of these sites after infection.

Bone Marrow Stroma

Studies by Hume et al. (12) indicated that murine adult bone marrow stroma contains a strongly F4/80+ population of stellate mature Mϕ at the center of islands of hemopoietic cells and distinct from rounded, weakly F4/80+ cells (Figure 1 A). Crocker and Gordon (16) have recently isolated intact hemopoietic cell clusters which contain these Mϕ by collagenase digestion of bone marrow followed by unit gravity sedimentation (Figure 1 B). Resident bone marrow Mϕ (RBMM) in clusters were further purified by adherence to glass coverslips (Figure 1 C) and stripped free of associated erythroid and myeloid cells by gentle pipetting (Figure 1 D). Table II summarizes the expression of various markers by these cells, compared with resident peritoneal Mϕ (RPM). Isolated RBMM sometimes display long F4/80+ plasma membrane processes to which immature erythroid and myeloid hemopoietic cells bind avidly. These Mϕ do not proliferate (Figure 1 E), unlike the immature, smaller weakly F4/80+ promonocytes also found in these preparations, which respond to CSF-1 and represent newly developing Mϕ. Apart from F4/80, RBMM express several other Mϕ markers including FcR, Mannosyl, fucosyl Receptors MFR, and characteristic cytochemical properties such as acid phosphatase. Although a subpopulation of RBMM strongly express Ia Ag, RBMM are not activated and do not exhibit a respiratory burst in spite of vigorous uptake of zymosan (Figure 1 F), unlike monocytes. RBMM do not express Mac-1 Ag or CR3 activity, but bear a novel surface hemagglutinin, which mediates rosette formation with unopsonized sheep erythrocytes (E). This lectinlike erythrocyte receptor (ER) is also found on a proportion of Mϕ obtained by collagenase digestion of liver, lymph nodes, and spleen, but is virtually absent from peritoneal Mϕ (resident, elicited, or activated) and is undetectable on circulating monocytes and non-Mϕ. Recent studies indicate that the ER can be induced on monocytes or Mϕ that lack this receptor by continuous exposure to normal mouse plasma or serum (Crocker, P., unpublished observation).

It is not known whether sheep erythrocytes bind to the same Mϕ

Table II. Comparison of Phenotype of Resident Bone Marrow and Peritoneal Macrophages from C57B1/6 Mice

	RBMM	*RPM*
Surface antigens		
F4/80	+ + +[c]	+ +
M1/70 (Mac 1, CR3)	–	+ +
2.4G2 (FcR IgG1/2b)	+ + +	+ +
M5/114 (Ia)	+ + (20-60%)	+ (5-20%)
7/4	–	–
Surface receptors		
Zymosan		
Phagocytosis	+ + +	+ +
Respiratory burst	–	+ / –
FcR IgG2a	+ + + (80%-	+ (25-
FcR IgG2b	+ + + 100%)	+ 40%)
MFR (mannan-inhibitable)	+ +	+ +
Complement[a]	–	+ +
Sheep erythrocytes[b]	+ + + (50-90%)	–
Histochemistry		
Acid phosphatase	+ +	+ +
Nonspecific esterase 1	+ +	+ +
Peroxidase	–	–
ATPase	+ +	+ +
Alkaline phosphatase	–	–

Adapted from Crocker and Gordon 1985 (16).

a. Background erythrocyte binding taken into account.

b. Scored on binding more than four erythrocytes.

c. More than 95% of cells positive unless otherwise indicated. – , negative; + , weak; + + , moderate; + + + , strong.

receptor as do immature hemopoietic cells (erythroid, myeloid, or monocytic). Bound E are rarely ingested and the bulk of immature hemopoietic cells remain fully viable at the Mϕ surface, although phagocytic debris, presumably derived from ingested erythrocyte nuclei and altered/damaged cells, is present in RBMM. Many of the bound hemopoietic cells proliferate vigorously on the surface of RBMM (Figure 1 E) and there

is enrichment of intermediate stages of development, but not progenitors, in the clusters. These studies imply that there is precise control by RBMM of enhanced growth and differentiation of bound hemopoietic cells versus their uptake and destruction. Activation of ingestion could involve appearance of new/altered ligands, collaboration with additional Mϕ receptors or activation of lectinlike ER, as described for CR3 (22).

Kupffer Cells

Isolated mouse Kupffer cells (KC) (Figure 3C) express several Mϕ markers, including F4/80, FcR, and ER, but lack Mac-1 (14). They can be distinguished from liver endothelial cells which also express an FcR for IgG1/2b subclasses and MFR by the presence of Ag F4/80 and an additional FcR for IgG2a ligands (23). Lepay et al. (24) have shown that resident KC are selectively refractory to the respiratory burst priming action of interferon-gamma and lack the ability to restrict growth of *Listeria monocytogenes*. After infection by *L. monocytogenes* (24) or *P. yoelii* (14), there is a large influx into the liver of Mϕ that express high respiratory burst, microbicidal and parasiticidal activities (Table III). The Mϕ responsible for clearance of these infections display a common activation phenotype (high respiratory burst, low MFR) to that induced by other organisms or parasites which induce cell-mediated immunity, e.g., BCG, *Toxoplasma brucei* (25), and similar cells can be isolated from different sites such as liver (14), spleen (14), or peritoneal cavity (25), depending on the organisms. The ease with which resident and recruited cells can be distinguished after isolation depends on the kinetics of infection and on the particular agent, as noted.

Spleen

In normal mice F4/80 labeling in situ is most striking in red pulp, with few F4/80+ Mϕ in T cell-dependent areas, except where arterioles penetrate the white pulp (12). Marginal zone Mϕ express other leukocyte Ag (7) such as Mac-1 and may also bear unique markers (18, 26). After collagenase digestion, spleen Mϕ isolated by adherence display a heterogeneous phenotype, and further single-cell analysis is needed to relate the expression of particular Ag and other markers to the properties of cells from different regions of the spleen. In our hands (14), spleen adherent Mϕ express relatively high Ia, fibrinolytic and respiratory burst activities, but low MFR, compatible with cell immaturity resulting from local hemopoiesis or partial activation of Mϕ. This population can be greatly expanded by *P. yoelii* infection, with further sharp decrease in MFR activity, an indication of immune activation.

Table III. Comparison of Resident Mouse Kupffer Cells and Liver Macrophages Freshly Isolated from C57 B1/6 Mice 8–12 Days after Plasmodium yoelii Infection

Property	*Kupffer cells*	*Malaria-induced liver Mϕ*
Yield	2×10^6/g liver	$2.5\text{–}5.0 \times 10^7$/g liver
Morphology	Homogeneous	Heterogeneous, enhanced spreading, endocytic debris, pigment laden
Surface		
Ag F4/80	+	+
Mac 1	0	Enhanced
7/4	0	Enhanced
Ia	±	±
FcR (IgG1/2b)	+	+
FcR (IgG2a)	+	+
MFR	+	Reduced
Sheep erythrocyte receptor	+ (25%)	+ (35%)
Secretory		
PA	0	Enhanced
O_2^- (PMA)	0	Enhanced

Adapted from Reference 14. Liver endothelial cells in the same adherent preparations were F4/80 – , FcR (IgG1/2b) + , FcR (IgG2a) – , ER – , and MFR + (activity also reduced by *P. yoelii* infection). Malaria-induced blood monocytes/Mϕ and spleen-adherent Mϕ showed very similar properties to those isolated from liver.

Langerhans Cells

In the mouse these cells express several Mϕ markers in situ, e.g., F4/80 Ag (Figure 2 A) (12), FcR (21), and ATPase (27) activity. However, the relationship between Langerhans cells, Mϕ, and Steinman-Cohn dendritic cells from spleen, which are F4/80– (28), is not clear. Recently, Schuler and Steinman (21) have shown that after isolation from epidermis, Langerhans cells lose F4/80 Ag and acquire the ability of dendritic cells to stimulate mixed leukocyte reactions. Similar F4/80+ cells are present in other complex epithelia in the mouse, e.g., cervix (29). There may

be substantial differences between species because human epidermal Langerhans cells do not express several Mϕ Ags. The phenotype of human Langerhans cells may indicate that these bone marrow-derived cells diverged from Mϕ earlier during differentiation or that modulation of cell phenotype can occur in the periphery, depending on the local environment. Dedifferentiation of bone marrow stromal Mϕ in the absence of a specific inducer in mouse serum (Crocker, P., unpublished observation), highlights the importance of cell culture artifacts in phenotypic variation. Absence of F4/80 Ag on osteoclasts (30), another bone marrow-derived population of cells that express some, but not other, Mϕ markers poses a similar problem of lineage interrelationships. Finally, the dendritic morphology of Langerhans cells, RBMM, and microglia underlines the importance of local environment in Mϕ differentiation and the need to analyze multiple markers.

Origin and Functional Significance of Macrophage Heterogeneity

Our current information thus makes it possible to distinguish several phenotypes for Mϕ in the periphery. The distinction between resident, elicited, and immune-activated Mϕ initially based on studies with peritoneal cells can now be extended to cells from other sites (cf. Tables I and III). Elicited/activiated Mϕ with similar features can accumulate in various organs in response to different agents, and there is no evidence that these cells differ according to the site of localization. The presence of particular cells such as hepatocytes in their local environment could result in unique local interactions.

Among resident Mϕ in the animal there may be different populations of "free" (often rounded) or "fixed" (usually stellate) cells. Expression of specific surface molecules such as the ER will facilitate classification of Mϕ in tissues. Sinus-lining "endothelial" Mϕ (e.g., in liver [29], lymph nodes [12], adrenal and pituitary glands [31]) could differ from Mϕ that have migrated from vessels, plasma, and other cells in the circulation. Mϕ embedded in different tissues display common as well as unusual features. Thus, many share a stellate or spindly morphology, display a low rate of turnover (16) and express high F4/80, with low Mac-1 Ag (16, 32). As noted, regional differentiation is evident in the central nervous system (microglia) and skin (Langerhans cells) and may be most marked in lymphoid organs and bone if dendritic cells, osteoclasts, and some Mϕ are mononuclear phagocytes which lack F4/80.

There is no evidence at present that these and other functional and

regional differences arise by clonal diversification of Mϕ. Independent clones of Mϕ derived from bone marrow progenitors after cultivation in the presence of CSF-1, with or without lymphokine, show no interclonal differences in expression of markers such as F4/80, Mac-1, or Ia (6, 33). It is likely that Mϕ heterogeneity arises from variation in maturation and as a result of local modulation by cytokines (CSF-1, GM-CSF, Il-3, G-CSF), interferons (α, β, γ), hormones (glucocorticoids, prostaglandins), and microbial products (MDP, lipopolysaccharides). The influence on Mϕ of various plasma-derived and matrix components (fibronectin, collagen, fibrin, laminin) and products of other cells (e.g., platelets, fibroblasts, etc.) needs further study, as does the effect of local environmental factors such as reduced oxygen tension. Many of the phenotypic features of activated Mϕ can be induced in nonactivated Mϕ by lymphokines and interferon-gamma, but the role of Mϕ maturity in determining the response to these agents has not been defined. The phenotype of Mϕ could also be modified by T-cell products other than interferon-gamma and by T cell-independent activators of Mϕ, including peptides derived from antibody or the plasma protein cascades (complement, coagulation, fibrinolysis, kinins). The role of various cytokines and other immunomodulatory molecules in expansion of Mϕ numbers and cell recruitment in vivo is still largely unknown.

We have emphasized defense functions in which oxidative killing plays a major role and compared the poor respiratory burst activity of resident Mϕ in liver and bone marrow with the enhanced activity of Mϕ recruited by organisms which evoke cellular immunity. Other effector functions including nonoxidative killing could show overlap among different Mϕ populations. For example, resident Mϕ in liver may determine innate resistance to infectious agents such as hepatitis virus or *Leishmania.* The distribution of resident Mϕ populations along portals of entry (gut, skin, lung) or at vascular interfaces (sinusoids, choroid plexus) suggests that these cells play an important role in first-line defense. Resident peritoneal Mϕ secrete complement proteins of the alternative pathway which locally opsonize targets in their vicinity and promote phagocytic uptake via CR3, in conjunction with MFR (34, 35). Resident peritoneal Mϕ can also generate high levels of prostaglandins which with other products (complement, platelet-activating factor) may initiate an inflammatory reaction. Elicited or activated Mϕ lose the ability to secrete some of these mediators, but release high levels of other products including toxic oxygen products, neutral proteinases, and cachectin/tumor necrosis factor, which mediate cytotoxicity and regulate connective tissue, plasma protein catabolism, and metabolic activities in target cells (36).

Recent studies of Mϕ from blood or peritoneal cavity have shown that Mϕ express specific plasma membrane receptors for many different ligands (37) and synthesize and secrete a wide range of products acting locally or at a distance. However, there is very little information on which surface or secretory molecules are produced in tissues or how these potential Mϕ products are altered by cell recruitment and stimulation. Many of these receptors (e.g., for Ab, complement, sugars, fibronectin, modified lipoproteins, cytokines, peptides) control Mϕ endocytic and metabolic activity and Mϕ products (e.g., interleukin 1, erythropoietin, granulocyte/macrophage-colony-stimulating factor, fibroblast growth factors, apolipoprotein E) in turn control the growth, differentiation and biosynthetic activity of cells in their vicinity. Macrophage receptors and products are able to contribute to the metabolism of plasma proteins (liver), hormones (adrenal gland, ovary, testis), neurotransmitters (central and peripheral nervous system), and vasoactive peptides (gut, kidney), and the synthesis and turnover of basement membranes (epithelia) and connective tissue during embryogenesis, tissue remodeling, inflammation, and repair. Macrophages could control the growth of hemopoietic, lymphoid and endothelial cells, fibroblasts and keratinocytes either by producing growth factors or by influencing the ability of various target cells to respond to these or other growth factors. Surface molecules that mediate specific Mϕ-cell interactions could regulate the responses of various target cells and the secretory activities of Mϕ themselves. In the light of these considerations it is clear the Mϕ defense functions include a range of tissue homeostatic and trophic functions, as well as phagocytosis and target destruction. Further studies will indicate whether these dual functions can be performed by the same cell populations and the same Mϕ.

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Genetic Control of Monocyte Production and Macrophage Functions

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Introduction

"Mononuclear phagocytes" is the name given to a cell line that is composed of monoblasts, promonocytes, monocytes, and macrophages. The dividing cells of this cell line, i.e., the monoblasts and promonocytes, reside in the bone marrow. The monocytes leave the bone marrow and are transported via the peripheral blood to the tissues and body cavities, where they become macrophages. On the basis of what is known about the origin of these cells, together with similarities in their morphologic, cyto- and immunochemical, and functional characteristics, it has been proposed that these cells comprise a system, which is called the mononuclear phagocyte system (1).

This chapter includes a short review dealing with (*a*) the characterization of cells, (*b*) the present views on the origin and kinetics of mononuclear phagocytes and the humoral regulation of monocyte production, and (*c*) in particular, the genetic control of monocyte production during an inflammatory response and of macrophage functions.

General Aspects of Mononuclear Phagocytes

Characterization

The physiologic functions of mononuclear phagocytes and their role in pathophysiology cannot be studied without adequate characterization of the cells under investigation. Until recently, characterization was based mainly on morphologic criteria, but this is not sufficient, because mononuclear phagocytes appear in many different forms. More advanced techniques must therefore be used to characterize a cell as a mononuclear phagocyte and to distinguish such cells from other types of cells of similar morphology.

The most reliable markers for the identification of mononuclear phago-

cytes of human or animal origin are nonspecific esterase (with α-naphthyl butyrate or acetate as substrate) (2, 3) and lysozyme, which can be demonstrated by the use of an immunofluorescent and immune peroxidase method and a (species-specific) antilysozyme antibody (3). As a marker, peroxidase is only useful in distinguishing between various developmental stages of mononuclear phagocytes, because the localization of peroxidase is different in monoblasts, promonocytes, monocytes, and macrophages; granules are positive only in monoblasts, promonocytes, monocytes, and exudate macrophages, whereas resident macrophages are negative (2, 3).

The use of monoclonal antibodies has greatly facilitated the characterization of cells, but only a few such antibodies are available for studies on mononuclear phagocytes. For murine cells, there is an appropriate antibody (F4/80) that stains monocytes and macrophages (4), and for human cells there are several reliable antibodies for blood monocytes, but not for macrophages (5). Antibodies that recognize monocyte precursors in the bone marrow are not yet available.

Receptors for the Fc part of IgG and for the third component of complement (C3) on the cell surface, and certain functional characteristics including endocytosis, are specific properties of mononuclear phagocytes (2). These cells carry Fc and C receptors in all stages of their development, but in immature stages (monoblasts and promonocytes), the percentage of cells with receptors is lower than that for monocytes and macrophages (2).

Whether a cell ingests opsonized bacteria or IgG-coated red cells (immune phagocytosis) is the most important criterion that must be fulfilled before that cell can be called a mononuclear phagocyte. However, ingestion of IgMc-coated red cells does not occur unless macrophages are activated (6).

Among the criteria for discrimination, pinocytosis is not very reliable: although all mononuclear phagocytes pinocytose avidly (2), pinocytosis is performed by other kinds of cell as well (e.g., fibroblasts), albeit less actively.

The minimal requirements to be satisfied before a cell can be called a mononuclear phagocyte are difficult to define. It is generally accepted that mononuclear phagocytes must react positively to at least esterase and lysoszyme, have Fc receptors in their membrane, and ingest IgG-coated particles. However, because of differences in their developmental stage and state of activation, not all cells satisfy a given criterion. For practical purposes, it is generally accepted that positivity of 90% or more of the cells in a population is sufficient to call a population positive for the criterion in question.

Origin and Kinetics

All hemopoietic cells originate from the pluripotent stem cell in the bone marrow. Cells derived from the pluripotent stem cell are committed stem cells. There is evidence pointing to the existence of a common stem cell committed to the mononuclear phagocyte and the granulocyte cell lines. Whether this stem cell gives rise to a stem cell committed only to form mononuclear phagocytes is not known but seems very likely. The most immature cell to be recognized as a mononuclear phagocyte is the monoblast, which has a cell cycle that lasts about 12 hours and, after division, gives rise to two promonocytes (Figure 1) (7, 8). The promonocyte, too, divides only once (cell cycle lasting about 16 hours) and gives rise to two monocytes (Figure 1) (9, 10). Thus, from monoblast to monocyte, there is a fourfold amplification. Monocytes do not divide further and leave the bone marrow randomly within 24 hours after they are formed. Mono-

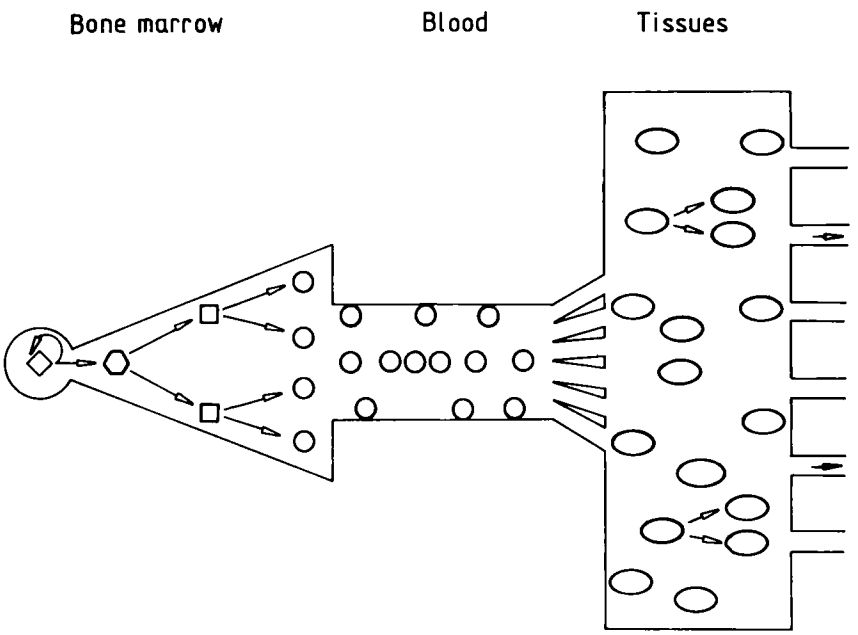

Figure 1. Schematic representation of current views on the origin and kinetics of mononuclear phagocytes. A stem cell (◇) gives rise to the monoblast (◯) which divides once, giving rise to two promonocytes (□); division of one promonocyte gives rise to two monocytes (○). The monocytes migrate from the bone marrow to the circulation and are distributed over a circulating and a marginating pool. Peripheral blood monocytes migrate to the tissues and body cavities where they differentiate into macrophages. Some of the macrophages derive from locally dividing mononuclear phagocytes. The ultimate fate of the macrophages (e.g., local death or migration to other sites) is uncertain.

cytes leaving the bone marrow are distributed over a circulating pool and a marginating pool, the latter accounting for about 60% of the total number of blood monocytes (11) (Figure 1). Monocytes remain in the circulation for a relatively long time (half-time about 17 hours) (12) compared with granulocytes (half-time about 7 hours), and leave this compartment randomly.

During the last 20 years, considerable attention has been given to the origin of macrophages. It is now generally accepted that monocytes give rise to macrophages under normal steady-state conditions (13) as well as during various kinds of inflammation and immunologic reactions. The question whether macrophages also divide locally in tissues and body cavities has been addressed again. In vitro labeling studies with the DNA precursor [^{3}H]thymidine showed a labeling index of 0.5–5% for macrophages at various sites (14), but it was found that these cells are not resident macrophages but cells that have very recently (less than 24–48 hours before dividing) arrived in the tissues from the bone marrow. Our thinking with respect to the origin of macrophages has been modified recently, mainly because use of the computer has improved the calculations related to various aspects of cell kinetics (15). The current view is that, in the normal steady state, the maintenance of the population of macrophages in a tissue compartment depends on the influx of monocytes from the circulation and on local division of mononuclear phagocytes that derive from the bone marrow and divide once in the tissues. The results of these calculations done with data obtained from kinetic studies in normal mice have shown that, of the monocytes leaving the circulation, about 56% become Kupffer cells, about 30% become spleen macrophages, about 15% become pulmonary macrophages, and about 8% become peritoneal macrophages (16). Further calculation has shown that on average, 75% or more of the macrophage population is supplied by the influx of monocytes and 25% or less by local division of (immature) mononuclear phagocytes. According to these data, the calculated mean turnover time of macrophages in the tissues would be about 7–14 days, which is much shorter than the turnover time previously reported (16).

In the normal steady state, the combination of a constant influx of monocytes into tissues (where they become macrophages) and a constant local production of macrophages in the tissues implies a constant death of macrophages in the tissues and/or a constant efflux of macrophages from the tissue compartments. Almost nothing is known about this point, except that macrophages in all probability migrate to the local lymph nodes, where they remain, and that alveolar macrophages leave the body via air spaces.

Regulation of Monocyte Production under Steady-State Conditions

The rather narrow fluctuation of the number of mononuclear phagocytes in the blood and tissues under steady-state conditions attests to a fine-tuning mechanism regulating monocyte production and distribution. At the level of the committed stem cell, the proliferation of what are called colony-forming units in culture (CFU-C), which give rise to colonies of macrophages or granulocytes, appears to be dependent on the presence of a family of regulatory glycoproteins known as granulocyte-monocyte colony-stimulating activity (17–19). As a group, these colony-stimulating factors (CSFs) differ in molecular weight, physical properties, and biological activity. At least four separable CSF subclasses can be distinguished, and all four can be described in terms of the kind of mature cells to which they give rise in culture. One subclass stimulates macrophage production exclusively (20, 21); one stimulates neutrophilic granulocyte and macrophage production (22); one stimulates the production of neutrophilic granulocytes (23, 24), and the fourth stimulates eosinophilic granulocyte production (25, 26). The CSF subclass that stimulates macrophage production, i.e., CSF-1, is still the best characterized of the CSFs (Table I). Colony-stimulating factor type 1 occurs in urine, extracts of several tissues, and in media collected from cultures of various organs and cells, such as primary fibroblasts and a variety of murine and human cell lines (19, 20, 24, 27, 28). This factor is composed of two

Table I. Differences in Biological and Biochemical Characteristics of the Factor Increasing Monocytopoiesis and Colony-stimulating Factor Type 1

Characteristic	*FIM*	*CSF-1*
Stimulation of monocyte precursors		
In vitro	No	Yes
In vivo	Yes	Unknown
Chemotactic activity	No	Unknown
Cell line specificity	Yes	Yes
Species specificity	No	No
Origin	Macrophages	Fibroblasts
Chemical nature	Protein	Glycoprotein
Molecular weight	18,000–24,500	45,000–86,000

similarly charged polypeptide chains covalently bound by disulfide bonds, and its molecular weight ranges from 45,000 to 86,000, the variation being mainly due to differences in the degree of glycosylation of the polypeptide chains (21) (Table I).

A negative feedback factor of the mononuclear phagocyte cell line, possibly acting at the level of the committed stem cell (CFU-C), is prostaglandin type E_2 (PGE_2), which is produced by macrophages (29). Lactoferrin, which is released by mature granulocytes, may also be a negative feedback factor that has no direct effect on the mitotic activity of cells in the bone marrow but inhibits the constitutive production of CSF by macrophages in vitro (30).

The role described for CSF, PGE, and lactoferrin in the mitotic activity of immature mononuclear phagocytes, including the committed stem cells, is based solely on results of in vitro studies. The role of these factors in the control of monocytopoiesis in vivo remains unknown.

Regulation of Monocyte Production during the Initial Phase of Inflammation

When the number of macrophages needed at the site of inflammation is rather large, a regulatory mechanism acting at the level of the dividing mononuclear phagocytes in the bone marrow is required to trigger the production of more monocytes. Because this increased production occurs by acceleration of the rate of division of monoblasts and promonocytes (12), chemical signals influencing the rate of division of these monocyte precursors in the bone marrow should be demonstrable in the circulation at certain times during the inflammatory reaction. Two endogenous factors, i.e., the factor increasing monocytopoiesis (FIM) and the monocyte-production inhibitor (MPI), have been found to regulate monocytopoiesis in vivo during an inflammation (31, 32). The FIM that occurs in the circulation during the initial phase of the inflammatory reaction (33–35) is a protein with a molecular weight of about 20,000 that has no colony-stimulating activity, is cell lineage-specific but not species-specific, and stimulates the mitotic activity of the promonocytes and probably also proliferation of the monoblasts (34–36) (Table I).

Origin and Production of the Factor Increasing Monocytopoiesis

The appearance of the FIM in serum is correlated with the presence of a phagocytic stimulus, which indicates that the FIM is directly released by cells at the inflammatory site (31, 34). Lysates of tissue macrophages contained FIM, and the FIM content of these macrophages decreased after phagocytosis in vivo (31, 34). Under in vitro conditions, further-

more, the FIM was released by resident macrophages upon triggering with a phagocytic stimulus (37). Granulocytes did not respond. This release of FIM in vitro occurs in two phases: (*a*) during the initial phase, the FIM stored in macrophages is released, and (*b*) after that, macrophages only release newly synthesized FIM (37).

It is postulated that, in vivo, FIM is synthesized and secreted by macrophages at the site of inflammation and then transported via the peripheral blood to the bone marrow where it exerts its stimulatory action (Figure 2). Although the assay methods now available do not permit detection of FIM under steady-state conditions, it is conceivable that this factor also regulates monocytopoiesis under such conditions.

Regulation of Monocyte Production during the Second Phase of an Inflammation

After resident and newly arrived macrophages have eliminated the inflammatory stimulus, the increased monocyte production is no longer necessary. If this increased production in the bone marrow demands the

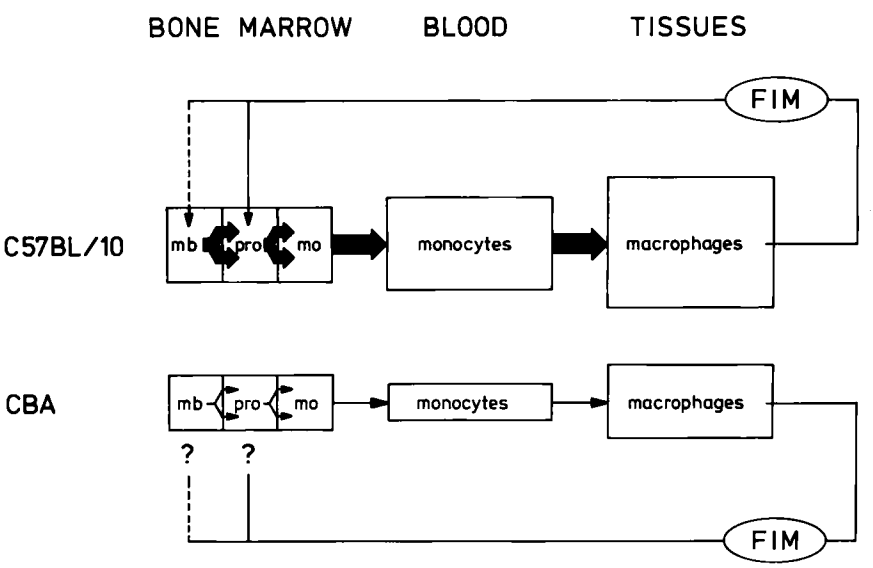

Figure 2. Schematic representation of the humoral regulation of the production of monocytes during an inflammatory reaction. In both C57BL/10 and CBA mice, FIM is produced and secreted by macrophages and is present in the circulation. In C57BL/10 mice, FIM stimulates monocyte (mo) production by interaction with promonocytes (pro) and monoblasts (mb); in CBA mice this interaction does not occur and therefore monocyte production is not stimulated.

continuous presence of FIM, termination of the increased FIM release by macrophages could occur by elimination of the stimulation of the macrophage membrane or the absence of phagocytosis by these cells. Actually the amount of FIM in serum is much lower in the second phase of the inflammatory response than in the first phase (34). However, the same effect can be achieved by factors capable of inhibiting FIM release, inactivating FIM, or counteracting the effect of FIM by a direct effect on monocyte precursors in the bone marrow.

Indications were obtained that the increased monocyte production is reduced by a factor appearing in the serum after the initial phase of the inflammatory reaction (i.e., MPI) (31). The monocyte-production inhibitor has a molecular weight of 50,000 or more, but has not yet been further characterized (31).

Role of Mononuclear Phagocytes in Genetically Controlled Resistance to Infection

Many host resistance factors are functional during an infection caused by intracellular pathogens, and each of them could be under separate genetic control. The differences in the mononuclear phagocyte system among mouse strains that vary in natural resistance to infection might be caused by: (*a*) a difference in the handling of the microorganisms (e.g., phagocytosis or intracellular killing) by the macrophages in the early phase of an infection, before the cellular and humoral immune responses reach an effective level, (*b*) a difference in the supply of exudate monocytes to the infected sites (e.g., influx of blood monocytes to the site of inflammation, or production of monocytes in and their release from the bone marrow). In addition, resident and exudate macrophages may differ in functional capacities reflecting the stages of macrophage differentiation.

In animal studies, use can be made of genetically divergent strains to study these mechanisms and evaluate their contribution to the resistance to infection by intracellular pathogens such as *Mycobacterium bovis*, *Salmonella typhimurium*, *Listeria monocytogenes*, and *Leishmania donovani* (38–41). Several recent reviews have dealt with the reduced lipopolysaccharide responsiveness and defective phagocytic ability of certain mouse strains (42), as well as the host defense against infections and tumors in selectively bred mice (41).

With respect to the natural resistance to intracellular pathogens, inbred mouse strains are generally characterized as either resistant or susceptible. This distinction is based on the ability of the host to control the early proliferation of bacteria in the spleen and liver. In the case of *S.*

typhimurium, resistance is due to the expression of at least one gene, which is autosomal, dominant, non-H2-linked, located on chromosome 1, and called Ity (43). The expression of the natural resistance of Salmonella is known to be conferred by bone marrow-derived cells (44), is sensitive to silica treatment (45), and does not require the presence of functional T lymphocytes (45). These findings imply a primary effector role for the mononuclear phagocytes.

Microbicidal Function of Macrophages of Genetically Different Mice

The mouse strains with inherited differences in resistance to viral and bacterial infection (i.e., the BSVS and BRVR strains) were among the first used to study some of the above points, i.e., in relation to the resistance to *S. typhimurium* (46, 47). Investigations on the early resistance to *S. typhimurium* showed that intraperitoneally injected Salmonella multiplied more rapidly in BSVS than in BRVR mice (48). In vitro, macrophages from BRVR mice killed *S. typhimurium* more effectively than macrophages from BSVS mice did. The macrophages of the two mouse strains did not differ as to their phagocytic activity. Differences in the microbicidal function of resident macrophages were therefore held responsible for differences in natural resistance.

Work done on inbred mouse strains that are more widely used in scientific research supported this conclusion (49, 50). We studied the genetically determined difference in natural resistance to *S. typhimurium* in susceptible C57BL/10 and resistant CBA mice (Table II). The total number of *S. typhimurium* recovered from the peritoneal cavity between 6 and 72 hours after intraperitoneal injection of the bacteria proved to be higher in susceptible C57BL/10 mice than in resistant CBA mice (51). Furthermore, in C57BL/10 mice the proliferation of *S. typhimurium* in the peritoneal cavity was more rapid than in CBA mice.

To find out whether these differences between C57BL/10 and CBA mice in bacterial proliferation in vivo reflect the function of their resident macrophages, we studied the phagocytosis and intracellular killing of Salmonella. In these studies resident macrophages of the two strains were equally efficient in phagocytosing opsonized *S. typhimurium* in vitro, but differed in their ability to kill the ingested microorganisms (50). The initial rate of intracellular killing of *S. typhimurium* by macrophages was determined during and after phagocytosis in vitro and after phagocytosis in vivo. Under all three conditions, consistent difference was found between macrophages of CBA and C57BL/10 mice, the initial rate of intracellular killing being about twice as high in the former (Table II). Thus, the differences between CBA and C57BL/10 mice in natural resistance

Table II. Differences in Some Biological Characteristics of C57BL/10 and CBA Mice

Biological characteristic	*C57BL/10*	*CBA*
LD_{50} for infection with		
L. monocytogenes	High	Low
S. typhimurium	Low	High
Activity of peritoneal macrophages in intracellular killing of		
L. monocytogenes	High	High
S. typhimurium	Low	High

to *S. typhimurium* was reflected in the functioning (i.e., the efficacy of intracellular killing) of their resident macrophages.

It is uncertain whether the genetically determined differences between macrophages of C57BL/10 and CBA mice are due to or supplementary to the Ity gene expression. Recently, the phenotypic expression of Ity and other resistance genes was studied in congenic mouse strains. In adherent cultured resident peritoneal macrophages from such mice, the Ity gene is apparently expressed as a bacteriostatic rather than bactericidal effect (49), although more recent findings indicate that there is also a difference in the initial intracellular killing of *S. typhimurium* by these macrophages (52). Similar findings concerning the microbicidal function of macrophages have been reported for the genes controlling the natural resistance of mice to infection by *Mycobacterium bovis* (53) and *Leishmania donovani* (54). It is noteworthy that these genes are either tightly linked with or identical to Ity (40).

The resistance pattern of CBA and C57BL/10 mice is reversed in infection by *Listeria monocytogenes*: CBA mice are susceptible and C57BL/10 mice are resistant (Table II). However, assessment of the intracellular killing of Listeria revealed similar rates of intracellular killing by resident macrophages of both strains (Table II). These results indicate that other host defense mechanisms must play a more important role in *L. monocytogenes* infection. One such mechanism related to the mononuclear phagocyte system could be the magnitude of the inflammatory reaction, as expressed in the increase in number of exudate macrophages (55, 56).

Table III. Differences in inflammatory responses of C57BL/10 and CBA Mice

Inflammatory responses	*C57BL/10*	*CBA*
Increase in the number of		
blood granulocytes	Low	High
peritoneal granulocytes	High	Low
blood monocytes	High	Absent
peritoneal macrophages	High	Low
Production of FIM	High	High
Stimulation of monocyte production by FIM	High	Low

Genetic Control of the Inflammatory Response

Inbred mouse strains can be roughly divided into two groups which differ by a factor of approximately 100 with respect to the 50% lethal dose of *L. monocytogenes*, C57BL/10 mice being more resistant than CBA mice (25). This substantial difference in resistance proved to be due to a single autosomal gene called Lr (40, 57).

Genetic linkage was recently reported to exist between the level of natural resistance to infection by *L. monocytogenes* and the magnitude of the increase in the number of exudate macrophages (55). It was not clear, however, how this Lr gene regulates the macrophage inflammatory response. Because macrophages themselves regulate the supply of monocytes during an inflammation and can increase it be accelerating the production of monocytes in the bone marrow via the secretion of FIM, it seemed possible that the level of gene control might lie at the production of FIM or the sensitivity of monocyte precursors to this protein. During a sterile inflammation, Listeria-resistant C57BL/10 mice showed a strong increase of the number of exudate macrophages and blood monocytes, whereas Listeria-susceptible CBA mice did not respond in this way (55, 56) (Table III). Comparable differences were found for the supply of granulocytes (58–62) (Table III). In this initial phase of the inflammatory reaction, FIM production and release were roughly the same as to time course and degree in both mouse strains (56, 61) (Table III). This finding excluded the possibility that the presence of FIM is the limiting

factor. The main difference between the two mouse strains is, however, that C57BL10 mice respond to injection of FIM with monocytosis and CBA mice do not (56, 61). Because an increase of the number of monocytes after injection of FIM is the reflection of increased proliferation of promonocytes and probably also of monoblasts, it may be concluded that only C57BL10 mice are able to respond to FIM by increased production of monocytes (Figure 2). This makes it conceivable that the Lr gene controls resistance to listeriosis via a divergent sensitivity of monocyte precursor cells to FIM. It is possible that a role is played here by CSF-1, which, on the basis of its biochemical and biological characteristics, is not identical to FIM (Table I). In a recent study, however, it was found that *L. monocytogenes* infection induces significant increases in CSF in both Listeria-resistant and Listeria-susceptible mouse strains (63). The level of colony-stimulating activity rose higher in the Listeria-susceptible mouse strains, probably as a result of the rapid bacterial proliferation in these strains of mice, which leads to high bacterial numbers. Thus CSF is not a limiting factor. Because colony-stimulating cells of both mouse strains were found equally sensitive to the action of CSF, these findings raise questions concerning the role assigned to CSF with respect to the resistance and susceptibility to *L. monocytogenes*. Of more importance in this respect would seem to be the finding that higher numbers of colony-stimulating cells are present in the bone marrow and spleen of resistant mice than of susceptible mice, but nothing conclusive is known about this as yet.

There is also a difference in the number of blood granulocytes of C57BL/10 and CBA mice during the early phase of an inflammatory response (Table III). Because the response to FIM is cell lineage-specific, i.e., there is no effect on the numbers of blood lymphocytes and blood granulocytes (56, 61), the greater supply of granulocytes to the lesion cannot be explained by a bone-marrow response to FIM. In the early phase of the response, the extra supply of granulocytes can be drawn from the pool in the vascular system and the storage pool in the bone marrow, but a sustained demand for granulocytes requires increased production (64). In this respect, it is noteworthy that similar differences in the increase of granulocytes and exudate macrophages at the inflammatory site after injection of thioglycollate broth and heat-killed *L. monocytogenes* were recently found between mouse strains that differ as to the level of activity of the fifth complement component (C5) (58, 60, 65). Regulation of these divergent inflammatory responses has been ascribed to the Hc gene, which controls the synthesis of complement factor C5. For C57BL/10 and CBA mice, this possibility can be excluded, because these strains have similar

levels of C5 (61), and it is therefore very unlikely that there is a difference in the formation of the very potent chemotactic factor C5a. Thus, there might be a difference between the response of C57BL/10 and CBA mice to other factors that stimulate the production and/or release of granulocytes.

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The *Legionella pneumophila* Model

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Introduction

Legionella pneumophila is the causative agent of Legionnaires' disease, a form of pneumonia that can be severe and fatal (1, 2). The organism also causes a milder and distinctly different febrile illness without pneumonia termed Pontiac fever (3). A gram-negative bacterium, *L. pneumophila* is a facultative intracellular parasite that multiples in human monocytes and alveolar macrophages, various animal macrophages, and a variety of cell lines (4, 5). The bacterium does not multiply in polymorphonuclear leukocytes, tissue culture medium, conditioned medium, or serum, but does multiply on artificial media (4).

Infection of human mononuclear phagocytes by *L. pneumophila* is an excellent in vitro model for studies of intracellular parasitism, macrophage activation, and cell-mediated immunity (Table I). The model offers important advantages over other models used for these purposes. First, in contrast to *Listeria monocytogenes*, *L. pneumophila* multiplies exclusively intracellularly under tissue culture conditions, allowing study of intracellular interactions independent of extracellular ones. Second, multiplication of *L. pneumophila*, in contrast to *Trypanosoma cruzi*, *Toxoplasma gondii*, chlamydiae, and rickettsiae, can be quantitated accurately by colony-forming units. Third, in contrast to *Mycobacterium tuberculosis* or *Mycobacterium leprae*, *L. pneumophila* multiplies rapidly in vivo and in vitro allowing experiments to be concluded in a relatively short time. Fourth, in contrast to obligate intracellular pathogens (e.g., *T. gondii*, *M. leprae*), *L. pneumophila* multiplies on artificial media and to high levels, making it easy to obtain large numbers of pure organisms for biochemical analysis, antigen purification, electron microscopy studies, etc. Fifth, in contrast

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Table I. Advantages of the Legionella pneumophila Model

1. Multiplication is exclusively intracellular under tissue culture conditions
2. Multiplication is quantifiable in colony-forming units
3. Multiplication is rapid
4. Multiplication occurs on artificial media and to high levels
5. Organism is easily visualized by light and fluorescence microscopy
6. A good and inexpensive animal model exists in the guinea pig
7. Organism can be stored for a prolonged period at − 70°C without significant loss of viability or infectivity

to rickettsiae, *L. pneumophila* can be easily visualized by light and fluorescence microscopy. Sixth, a good and relatively inexpensive animal model for *L. pneumophila* is available in the guinea pig, allowing parallel in vivo and in vitro studies. Seventh, the organism can be conveniently stored at −70°C without significant loss of infectivity or viability.

This paper broadly describes the *L. pneumophila* model. The paper reviews (*a*) humoral and cellular immunologic responses of humans to the bacterium, as reflected by in vitro studies; (*b*) the influence of antibiotics on intracellular *L. pneumophila*; (*c*) the unusual way *L. pneumophila* is phagocytized by human phagocytes; and (*d*) various intracellular phenomena that characterize *L. pneumophila* interactions with mononuclear phagocytes and that appear to have general relevance to intracellular parasitism.

Humoral Immunity

Patients with Legionnaires' disease respond to the infection by producing antibody to *L. pneumophila*. Virtually all of this antibody (>98%) is directed against the lipopolysaccharide of the bacterium (6). Very little antibody is directed against the other major component of the bacterial surface, the major outer membrane protein, which is a porin (6, 7).

Anti-*L. pneumophila* antibody from patients is not bactericidal, i.e., it

does not promote complement lysis of the bacteria. *Legionella pneumophila* is completely resistant to the bactericidal effects of human serum in the presence or absence of high-titer human or rabbit anti-*L. pneumophila* antibody (6).

In the presence of complement, human anti-*L. pneumophila* antibody markedly promotes phagocytosis of *L. pneumophila* by human polymorphonuclear leukocytes, monocytes, and alveolar macrophages (5, 8, 9). However, these phagocytes kill only a modest proportion (0.25–0.5 log) of the ingested bacteria. The surviving bacteria multiply in monocytes as rapidly as bacteria ingested in the absence of antibody (9).

Because antibody fails (*a*) to promote complement lysis of *L. pneumophila*, (*b*) to promote effective killing of *L. pneumophila* by professional phagocytes, and (*c*) to inhibit intracellular multiplication of *L. pneumophila*, humoral immunity probably does not play a major role in host defense against this pathogen.

Cell-mediated Immunity

Patients with Legionnaires' disease also develop a specific cell-mediated immune response to *L. pneumophila*. Lymphocytes from patients who have recovered from Legionnaires' disease respond to *L. pneumophila* antigens with proliferation and the production of cytokines capable of activating human monocytes (10). Human monocytes activated by such cytokines or by cytokines in the supernatants of mononuclear cell cultures sensitized to other antigens or the mitogen concanavalin A inhibit *L. pneumophila* intracellular multiplication (5). Human monocytes and alveolar macrophages activated with gamma interferon also inhibit *L. pneumophila* intracellular multiplication (N. Bhardwaj, T. W. Nash, and M. A. Horwitz, manuscript in preparation).

Activated monocytes inhibit *L. pneumophila* multiplication in two ways. First, they phagocytize ~50% fewer bacteria than nonactivated monocytes, thereby restricting access of the bacteria to the intracellular milieu that they require to multiply (11). Second, activated monocytes markedly slow the multiplication rate of bacteria that are internalized, increasing the doubling time threefold (11).

Activated monocytes do not have a greater capacity to kill *L. pneumophila* than nonactivated monocytes. As in the case of nonactivated monocytes, activated monocytes require anti-*L. pneumophila* antibody to kill any bacteria and even then, they kill only a small proportion of an inoculum (11).

These results indicate that in humans cell-mediated immunity likely

plays a major role in host defense against *L. pneumophila* as it does against other intracellular pathogens.

Influence of Antibiotics on Intracellular L. pneumophila

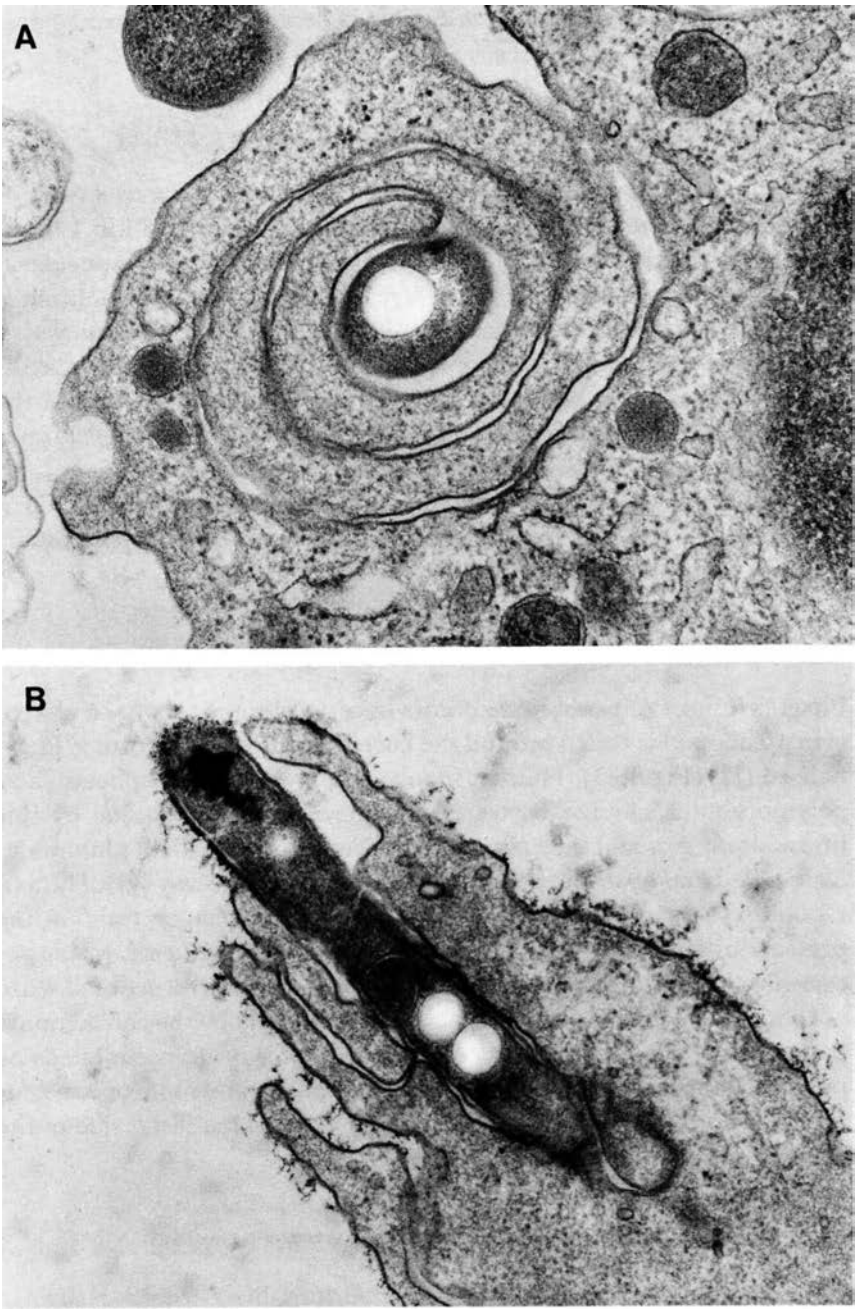
Erythromycin and rifampin are the drugs of choice for the treatment of Legionnaires' disease. These antibiotics rapidly inhibit (within 1 hour) intracellular multiplication of *L. pneumophila* in monocytes at concentrations that are comparable to those that inhibit extracellular multiplication of *L. pneumophila* in artificial medium (12). The antibiotics, even at very high concentrations, do not kill intracellular bacteria and by electron microscopy, the bacteria appear intact within phagosomes in the monocyte. In contrast, erythromycin and rifampin kill *L. pneumophila* multiplying extracellularly at low concentrations of antibiotic. Antibiotic inhibition of *L. pneumophila* intracellular multiplication is reversible. When antibiotics are removed from infected monocyte cultures, *L. pneumophila* resumes multiplication intracellularly.

Phagocytosis

Phagocytosis of *L. pneumophila* occurs by a novel mechanism—a phagocyte pseudopod is coiled around the bacterium as the organism is internalized (13) (Figure 1). Human monocytes, alveolar macrophages, and polymorphonuclear luekocytes all phagocytize *L. pneumophila* by this unusual process, and they phagocytize live, formalin-killed, glutaraldehyde-killed and heat-killed *L. pneumophila* in the same way (13). Human monocytes phagocytize *L. pneumophila* by "coiling phagocytosis" in the presence or absence of normal serum. However, when *L. pneumophila* are coated with human anti-*L. pneumophila* antibody in the presence or absence of complement, monocytes phagocytize the bacteria by the conventional pattern of phagocytosis, a process in which phagocyte pseudopods or micropseudopods appear to move circumferentially and more or less symmetrically about a particle until their tips meet at the distal side of the particle and fuse.

Formation of the L. pneumophila Phagosome

After entering monocytes by coiling phagocytosis, live *L. pneumophila* form a novel ribosome-lined phagosome in human monocytes and alveolar macrophages (5, 14). Formation of this phagosome takes place during the


A
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first 4–8 hours after infection and entails a remarkable sequence of cytoplasmic events in which first smooth vesicles, then mitochondria, and finally ribosomes surround the phagosome membrane (Figure 2). The ribosomes surround the phagosome at a distance of ~ 100 Å from the phagosome membrane. Once the ribosome-lined vacuole is formed, *L. pneumophila* begins multiplying. The bacterium remains within the ribosome-lined vacuole during replication and multiplies until the monocyte becomes packed full with bacteria and ruptures.

In contrast to the situation with live *L. pneumophila*, formalin-killed *L. pneumophila* are not surrounded by smooth vesicles, mitochondria, or ribosomes after entry into monocytes. Moreover, formalin-killed bacteria are rapidly ingested, and by 4 hours, few remain intact (14).

Inhibition of Phagosome–Lysosome Fusion

Some intracellular pathogens, such as *Chlamydia psittaci*, *M. tuberculosis*, and *T. gondii*, have the capacity to inhibit fusion between the phagosome and host cell lysosomes. This capacity may be important to their intracellular survival or multiplication.

The interaction between the *L. pneumophila* phagosome and monocyte secondary lysosomes has been investigated by prelabeling the lysosomes with thorium dioxide, an electron-opaque marker, infecting the monocytes with *L. pneumophila*, and examining the cells by electron microscopy to determine whether fusion has occurred, as evidenced by the presence of thorium dioxide in the phagosome (15). The interaction between the *L. pneumophila* phagosome and both primary and secondary lysosomes of monocytes has been studied by infecting monocytes with *L. pneumophila*, fixing the cells, staining the phagosomes for acid phosphatase, an enzyme found in monocyte lysosomes, and examining the cells by electron microscopy to determine whether fusion has occurred, as evidenced by the presence of acid phosphatase reaction product in the phagosome (15). Phagosomes containing live *L. pneumophila* do not fuse with primary or secondary lysosomes at 1 hour after entry or at 4–8 hours after entry

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Figure 1. Phagocytosis of *L. pneumophila*. (A) Monocyte ingesting a single *L. pneumophila*. The bacterium is located in the center of a coiled monocyte pseudopod. As is frequently the case, the bacterium contains a lucent fat vacuole (×34,800). (B) Monocyte ingesting a single *L. pneumophila* observed from a different perspective from that in panel A. The pseudopod coil is sectioned along its axis so that the bacterium is located between finger-like projections of monocyte membrane (×27,000). (Reprinted with kind permission of M. I. T. Press from Horwitz, M. A. 1984. *Cell.* 36:27–33.)

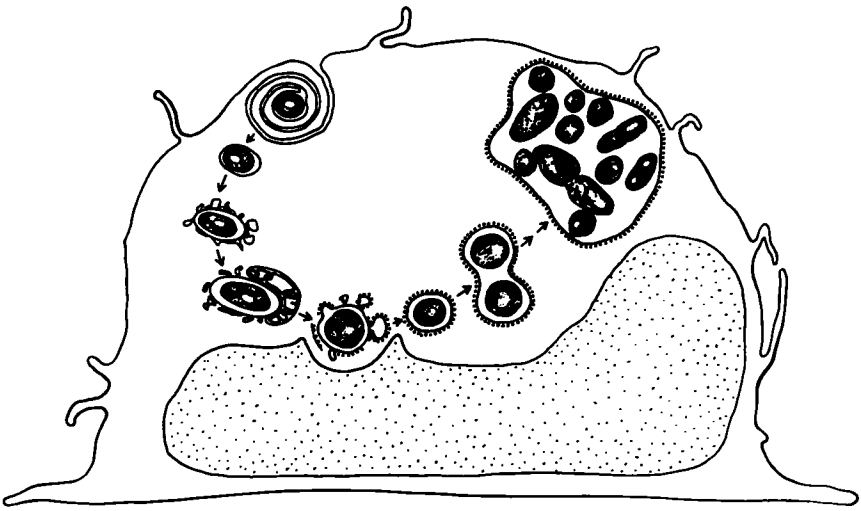

Figure 2. Diagram of the sequence of cytoplasmic events involved in formation of the *L. pneumophila* phagosome (*a*) *L. pneumophila* is coiled around the bacterium as the bacterium is internalized. (*b*) Immediately after phagocytosis, no cytoplasmic organelles are found surrounding the *L. pneumophila*-containing vacuole. (*c*) By 15 minutes after infection, the majority of *L. pneumophila* vacuoles are surrounded by smooth vesicles, aparently fusing with or budding off from the vacuolar membrane. (*d*) By 1 hour after infection, the majority of vacuoles are surrounded by at least one mitochondrion, closely apposed to the vacuolar membrane. Smooth vesicles are usually also present about the vacuole. (*e*) By 4 hours after infection, fewer smooth vesicles and mitochondria surround the vacuole, but now ribosomes and rough vesicles line the vacuole. (*f*) By 8 hours after infection, the *L. pneumophila* vacuole is studded with ribosomes. (*g*) The bacteria multiply within the ribosome-lined vacuole with a doubling time of ~2 hours. (*h*) The bacteria multiply until hundreds of organisms fill the vacuole. The monocyte becomes packed full with bacteria and ruptures. (Reprinted with kind permission of The Rockefeller University Press from Horwitz, M. A. 1983. *J. Exp. Med.* 158:1319–1331.)

by which time the ribosome-lined, *L. pneumophila* replicative vacuole has formed (15). In contrast, phagosomes containing formalin-killed *L. pneumophila* fuse with monocyte lysosomes by 1 hour after entry. Coating live *L. pneumophila* with antibody or with antibody and complement promotes fusion to a small degree, but the majority of phagosomes do not fuse (15). Similarly, activating monocytes promotes a small degree of phagosome–lysosome fusion (15). Erythromycin, a potent inhibiter of bacterial protein synthesis, has no influence on phagosome–lysosome fusion at a concentration of antibiotic that completely inhibits *L. pneumophila* intracellular multiplication.

Inhibition of Phagosome Acidification

Phagosomes containing particles that fuse with lysosomes are typically acidified to a relatively low pH soon after phagocytosis. Because *L. pneumophila* inhibits fusion, the pH of the *L. pneumophila* phagosome was studied to determine the extent to which it is acidified (16). The technique used to measure pH was quantitative fluorescence microscopy which involves labeling the bateria with fluorescein, allowing monocytes to ingest them, and exciting the fluorescein label on the bacteria with light of two different wavelengths (450 and 490 nm). Fluorescein has a pH-dependent excitation profile, and the pH of the phagosome can be determined from the ratio of fluorescence intensities resulting from excitation at the two different wavelengths.

The pH of phagosomes containing live *L. pneumophila* (6.1) is consistently higher by 0.8 pH units than the pH of phagosomes containing formalin-killed *L. pneumophila*. In contrast, there is no difference in pH between phagosomes containing initially live and killed *Escherichia coli*. Live *E. coli*, in contrast to *L. pneumophila*, are rapidly killed in monocytes, and like dead *E. coli*, they do not inhibit fusion and so enter phagolysosomes.

Live *L. pneumophila* do not induce a generalized inhibition of acidification. The presence of live *L. pneumophila* in one phagosome does not influence the pH of another phagosome containing an erythrocyte in the same monocyte. Phagosomes of very different pH exist simultaneously in the same monocyte (16).

Interactions of Live and Killed L. pneumophila with Mononuclear Phagocytes

Both live and killed *L. pneumophila* enter mononuclear phagocytes by a common mechanism, coiling phagocytosis, but thereafter their intracellular pathways diverge (Table II). Live *L. pneumophila* induces the formation of a novel ribosome-lined phagosome, inhibits phagosome–lysosome fusion, and inhibits phagosome acidification. In contrast, formalin-killed *L. pneumophila* does not form a specialized phagosome, inhibit phagosome–lysosome fusion, or inhibit phagosome acidification. The live organism multiplies in the ribosome-lined phagosome until it destroys the host cell. In contrast, the formalin-killed organism is rapidly degraded in the phagolysosome.

Table II. Interaction with Monocytes of Live and Killed Legionella pneumophila

	L. pneumophila	
	Live	*Formalin-killed*
Coiling phagocytosis	+	+
Formation of novel phagosome	+	–
Inhibition of phagosome–lysosome fusion	+	–
Inhibition of phagosome acidification	+	–

Table III. Common Features of Phagosomes Containing Legionella pneumophila, Toxoplasma gondii, and Chlamydia psittaci

	Phagosome surrounded by			*Inhibition of phagosome–lysosome fusion*	*Inhibition of acidification*
	Smooth vesicles	*Mitochondria*	*Ribosomes*		
L. pneumophila	+	+	+	+	+
T. gondii		+	±	+	+
C. psittaci		+		+	

Common Features of Phagosomes Containing Legionella pneumophila, Toxoplasma gondii, and Chlamydia psittaci

The sequence of events involved in formation of the *L. pneumophila* phagosome has not been described for other organisms. However, phagosomes of *T. gondii* and *C. psittaci* share some of the morphologic features of the *L. pneumophila* phagosome (Table III) (17, 18). The phagosomes of all three pathogens are surrounded at some point by mitochondria. Although *T. gondii* has not been reported to form a ribosome-lined phagosome as does *L. pneumophila*, at one stage, *T. gondii* phagosomes are surronded by a few strips of membrane-bound ribosomes. The phagosomes of these three organisms have more than morphologic features in common. All three organisms inhibit phagosome–lysosome fusion. In addition, *T. gondii* recently has been reported to share with *L. pneumophila* the capacity to

inhibit phagosome acidification (19). That the phagosomes of such disparate organisms share so many features suggests that a common mechanism underlies the capacity to form a specialized phagosome, inhibit phagosome–lysosome fusion, and inhibit phagosome acidification.

Conclusion

The *L. pneumophila* model offers an excellent and exciting opportunity to study details of the host immune response to an intracellular pathogen and mechanisms underlying both common interactions between host cell and pathogen (phagosome formation, inhibition of phagosome–lysosome fusion, and inhibition of phagosome acidification) and uncommon ones (coiling phagocytosis).

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Interferon-Gamma and Macrophage Activation in Cell-mediated Immunity

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Overview

Confronted by the complexity of the immune system, we sometimes lose sight of its primary function, defense of the host against infection. Without vigorous intervention, death from infection frequently ensues when any of the major classes of immune system cells is functionally or physically deleted by genetic error, disease, drugs, or radiation—T cells, B cells, polymorphonuclear leukocytes, or mononuclear phagocytes. Even antitumor immunity, of such importance to the aging individual accumulating genomic disarray, can be regarded as an offshoot of immune reactions shaped by evolution in response to infections that strike the young. The outcome of infections that run a prolonged course, during which cell-mediated immune reactions come into play, often devolves on how well macrophages respond to commands from lymphocytes to augment their antimicrobial capacity. To appreciate this, a previous generation of immunologists needed no lesson more dramatic than the baby born without a thymus (DiGeorge's syndrome). Today, acquired immunodeficiency syndrome drives the same message home: human T lymphotrophic virus III/lymphadenopathy virus (HTLV-III/LAV) cripples helper T cells, leaving otherwise normal macrophages unable to adapt to invasion by opportunistic microorganisms (1). The death of AIDS patients from bacterial, fungal, and protozoal infections despite aggressive antibiotic therapy testifies to the physiologic importance of the phenomenon called macrophage activation (2). We will define it here much as Mackaness did (3): enhancement, via immunologically specific reactions, of the nonspecific antimicrobial capacity of macrophages. It was only within the last 15 years that this process could be broken down

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into the following steps: T lymphocytes encountering specific microbial antigen release a soluble factor which activates macrophages for enhanced ability to kill the eliciting or antigenically unrelated microorganisms (4, 5).

Equally critical in defense against infection is the host's ability to heal wounds. This is another arena for the macrophage. In fact, the macrophage has two roles in this regard: removal of debris and promotion of tissue growth. However, killing of microorganisms and debridement of damaged tissue involve biochemical events that may not be conducive to the growth of vessels, connective tissue, and epithelia. For example, at least one class of macrophage antimicrobial products—the reactive oxygen intermediates—can damage tissue (6–8). At least two of the growth factors released by macrophages that promote proliferation of stromal cells—interleukin 1 (9) and tumor necrosis factor (TNF) (10, 11)—can also damage cells (10–13).

Thus, it is not enough to recognize that survival depends on the quantity and quality of mononuclear phagocytes that we can mobilize. We need to consider much more complex phenomena, such as coordination of the three chief anti-infectious functions of mononuclear phagocytes—killing of microbes, digestion of debris, and repair of tissue. There must be cells and factors—including, perhaps, products of macrophages themselves, resident in the tissues, beneath mucosae and around blood vessels—that can beckon and dismiss monocytes, call on the right elements of the inflammatory macrophage's repertoire at the right times, and augment or suppress each to the right extent. How these responses are regulated, as an epithelium is breached, infected, inflamed, sterilized, and healed, is almost completely unknown.

In fact, we have not even completed the catalogue of the cellular products that participate. To take a further step back, we are only beginning to appreciate the diversity of *cells* that can undergo immunologically mediated enhancement of their antimicrobial activity. At minimum, these include endothelial cells, fibroblasts, and hepatocytes. Interferon-gamma (IFNγ), for example, can enhance the ability of such cells to kill *Rickettsia* (14, 15), *Chlamydia* (16), *Coxiella* (17), *Toxoplasma* (18), and malaria (cited in Vilcek et al. [19]).

Finally, we are only beginning to understand the effector mechanisms induced during immunologically mediated enhancement of antimicrobial capacity. In the case of the macrophage, these fall into two groups: respiratory burst-dependent and respiratory burst-independent (20). Much has been learned about the former (21), yet not enough to answer these questions: Exactly how is the macrophage's capacity to produce superoxide and hydrogen peroxide regulated by cytokines? What is the nature of

the enzyme system that makes these products? Exactly how do they kill microorganisms? As for the respiratory burst-independent mechanisms of macrophages, all that can be said with assurance is that they exist. The antimicrobial mechanisms of cells other than phagocytic leukocytes are even more obscure.

From this skein of questions, one thread is picked up and traced in this chapter: steps leading to the identification of IFNγ as a major, physiologic macrophage-activating factor (MAF) (Table I). Even that path bristles with branches down which we have no time to venture. Thus, we will exclude consideration of macrophage interaction with the smallest pathogens (viruses) and the largest (multicellular helminths, nematodes, and trematodes). We will deal only with augmentation of antimicrobial activity rather than the equally fascinating topic of its suppression (22). And we will merely glance at evidence suggesting a role for MAFs other than IFNγ.

Presence of Interferon-Gamma in Lymphocyte-conditioned Media That Contain Macrophage-activating Factor

The capacity of an individual's lymphocytes to secrete IFNγ mounts in parallel with other manifestations of delayed-type hypersensitivity or cell-mediated immunity (23–26). In vitro, T cells of both helper and cyto-

Table I. Identification of Interferon-Gamma as a Mediator of Macrophage Activation

1. IFNγ is present in lymphocyte-conditioned media that contain MAF.
2. Monoclonal antibodies to IFNγ remove all detectable MAF from such media.
3. MAF in such media copurifies with IFNγ.
4. Pure, recombinant IFNγ (rIFNγ) is a potent MAF in vitro.
5. Among cytokines with effects on macrophages, MAF activity is relatively restricted to IFNγ.
6. Injection of low doses of rIFNγ in mice activates macrophages in vivo.
7. Injection of monoclonal antibody to IFNγ in mice prevents resolution of an infectious disease dependent on macrophage activation.
8. Injection of rIFNγ in cancer patients boosts the oxidative metabolism of their monocytes.
9. Injection of small doses of rIFNγ in lesions of lepromatous leprosy in man (where there appears to be a deficiency of endogenous IFNγ) elicits features of delayed-type hypersensitivity.

toxic phenotypes release IFNγ upon contact either with specific antigen or mitogens. Immunologically specific induction of IFNγ production satisfies one requirement for a mediator of macrophage activation as the term was defined above.

Natural killer (NK) cells are another source of IFNγ, although the physiologic stimuli for its release have not been fully defined. Noncytotoxic concentrations of H_2O_2 can trigger IFNγ secretion from NK cells (27), raising the possibility that phagocytes undergoing a respiratory burst during the ingestion of microorganisms in the early phases of infection might trigger the rapid release from NK cells of a signal directing the adaptation of macrophages to better handle the later phases. NK cells can accumulate in inflamed tissues (28), and thus might play a more important role in macrophage activation than has previously been considered. The ability to transfer the inducibility of macrophage activation with

Table II. Identification of the Lymphokine Enhancing Human Macrophage Oxidative Metabolism as Interferon-Gamma

		Macrophage H_2O_2 release	
Lymphokine preparation and means of induction	*Concentration before dilution, or specific activity*	*Ratio, experimental[a]/ control*	*% inhibition by anti-IFNγ MAb*
		mean ± SEM (no. exp.)	
Crude lymphokines (*U/ml*)			
Lentil lectin, mezerein	$0.1\text{–}1 \times 10^3$	3.8 ± 1.5 (6)	207[b] ± 43 (3)
Concanavalin A	$1.0\text{–}3 \times 10^3$	3.6 ± 0.5 (5)	86 ± 9 (5)
Toxoplasma antigen	$2\text{–}6 \times 10^3$	4.1 ± 0.5 (4)	108 ± 12 (4)
Purified native IFNγ (*U/mg*)			
lentil lectin, mezerein	10^6	7.2 ± 1.9 (4)	113 ± 8 (2)
staph. enterotoxin A	10^7	9.7 ± 2.4 (8)	91 ± 13 (5)
rIFNγ (*U/mg*)	3×10^6	17.7 ± 4.8 (14)	

From Nathan et al. (31).

a. Mean ± SEM (n) for 41 experimental sets = 643 ± 57 nmol H_2O_2/mg cell protein per hour.

b. Inhibition in excess of 100% probably represents unmasking of suppressive activity in the same preparation.

antigen-specific T cells (29) does not militate against a contribution of NK cells, because the factors that result in accumulation of NK cells in inflamed tissues might themselves be T cell-dependent. This issue needs further study.

Reports that macrophages themselves can release IFNγ (e.g., Robinson et al. [30]) await confirmation with a combination of reagents permitting definitive identification of the IFN and the cellular source.

Thus, it is no surprise that media conditioned by unseparated populations of lymphoid cells from blood (31, 32) or spleen (33, 34) in humans or mice, triggered with antigen or mitogen, contain IFNγ, often in abundance, together with MAF (Table II and Figure 1).

Harder to evaluate are reports of MAF-containing supernates that lack detectable IFNγ. These are usually the product of select cell lines, clones, or hybridomas. Monoclonality of cells by no means excludes pleiotypy of their secretory products, and IFNγ is one of the most common products of randomly selected T cell clones (35). Because assays for MAF are generally far more sensitive than those for the antiviral effect of IFNγ, IFNγ may sometimes be missed in MAF-containing clone-conditioned

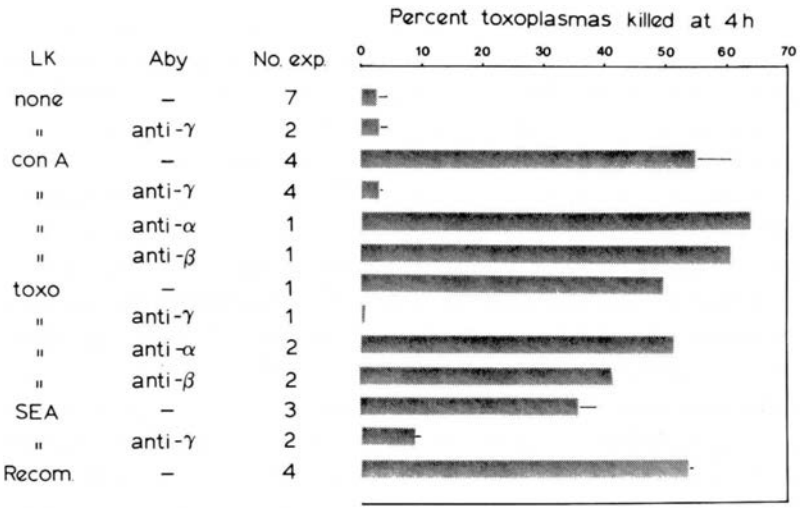


Figure 1. Toxoplasmacidal activity of human macrophages incubated in lymphokine preparations (*LK*) induced with concanavalin A (*con A*) or toxoplasma antigen (*toxo*), or in highly purified native IFNγ induced with staphylococcal enterotoxin A (*SEA*), or in pure rIFNγ (Recom), with or without antibody to the indicated classes of IFN. Incubations were in 100–300 antiviral U/ml of IFNγ for 2–3 days beginning 3–23 days after placing blood monocytes in culture. Means + standard error of the mean for the indicated number of experiments, each with cells from a different donor. Reprinted from Nathan et al. (31).

media (36) even when IFNγ is later found to account for the MAF in the same media (37). The existence of inhibitors of the antiviral action of IFNγ, which may not inhibit its other actions, further complicates the interpretation of apparent dissociations between the presence of IFNγ and MAF.

Neutralization of Macrophage-activating Factor by Monoclonal Antibodies to Interferon-Gamma

This has been reported with at least four antibodies in two species, where MAF was assayed by induction of antimicrobial activity against at least two protozoal and two bacterial pathogens, using either primary tissue macrophages or macrophages derived in vitro from monocytes, and using lymphocytes either from blood or spleen, stimulated with either antigens or mitogens (31–34, 34a). Some of the data are illustrated in Table II and Figure 1.

At face value, these observations argue that IFNγ and only IFNγ has MAF activity in a complex conditioned medium containing many cytokines. However, interpretation of these observations is more complex, and includes the following possibilities: another cytokine may cross-react with the antibodies; another cytokine may be an essential cofactor for the action of IFNγ, or vice versa; another cytokine may have potential for MAF activity but be present at too low a concentration to reveal it; another cytokine with MAF activity may be blocked by an inhibitor in the same supernate; another cytokine with MAF activity may have been produced but may be absent from the conditioned medium at the time of study because of lability during production, collection, or storage; or another cytokine with MAF activity may be absent from lymphocyte-conditioned medium because it is a product of other kinds of cells.

Not all monoclonal antibodies that neutralize the antiviral activity of IFNγ neutralize its ability to activate macrophages (34). The implications of this fascinating observation for the possible existence of functional domains of the IFNγ molecule and subclasses of IFNγ receptors on the same or different cells are still being explored. Thus, the failure of a monoclonal anti-IFNγ antibody to inhibit MAF in the fluid phase in a particular assay is not definitive proof that the MAF is different from IFNγ.

There is at least one report of residual MAF activity in lymphocyte-conditioned medium depleted of IFNγ with a monoclonal antibody (38). This activity, which enhanced the killing of *Leishmania* within macrophages, had no effect on macrophage antibacterial activity and thus may not conform to the description of macrophage activation as nonspecific in its

expression. The factor was added to macrophages after they had ingested the parasite, so that its possible direct antileishmanial activity (independent of effects on macrophages) remains to be excluded. In contrast, as already noted, when MAF was assayed by adding lymphocyte-conditioned medium to macrophages and then removing it before addition of *Leishmania*, all MAF could be inhibited by monoclonal anti-IFNγ antibody (33).

It has also been reported that monoclonal antibody to IFNγ removed all MAF from lymphocyte-conditioned media, as judged by induction of the ability to kill schistosomes, but left behind additional factors required for the optimal induction of schistosomulocidal activity by IFNγ at low doses (39). The identification of these additional factors will be of considerable interest.

Copurification of Macrophage-activating Factor in Highly Enriched Preparations of Native Interferon-Gamma

This observation (31, Table II, and Figure 1) makes it extremely unlikely that the monoclonal antibodies discussed above fortuitously cross-reacted with a cytokine physicochemically distinct from IFNγ. The point is strengthened by using two independent purification schemes for native IFNγ (31). However, it is not without precedent that a biologic effect attributed to one cytokine may be the result of a synergistic interaction with another cytokine that extensively copurifies. A classic example is IFNγ and lymphotoxin (40). Thus, the evidence up to this point supports the interpretation that IFNγ is necessary for macrophage activation in the assays used, but neither proves this nor establishes whether IFNγ is sufficient.

Association of Macrophage-activating Factor with Pure, Recombinant Interferon-Gamma

Since the initial reports (31, 32, Table III, and Figure 1), this observation has been extended to macrophage killing of at least 15 species of microbial pathogens (Table III), satisfying the second requirement of our definition of macrophage activation: that the antimicrobial activity induced be nonspecific in its expression.

The potency of rIFNγ as MAF is noteworthy. In some experimental systems, the half-maximal effective concentration is in the picomolar or subpicomolar range (31, 33), on the order of several hundred molecules per macrophage. This in itself argues that macrophage activation may be a physiologic function of IFNγ, more so than induction of antiviral activity or inhibition of host cell proliferation, which require considerably higher concentrations.

Table III. Nonviral Pathogens Whose Killing by Macrophages Is Enhanced by Interferon-Gamma

Parasite	*Notes*	*References*
Bacteria		
Rickettsia prowazekii	a	14
Chlamydia psittaci		41
Listeria monocytogenes		34, 42, 64
Mycobacterium intracellulare		44
Pneumophila legionella	*	
Salmonella typhimurium	b	45
Protozoa		
Toxoplasma gondii		31, 33, 34a
Leishmania donovani		32, 33
Leishmania major		38
Plasmodium falciparum	c	46
Trypanosoma cruzi		47, 48
Fungi		
Histoplasma capsulatum	a	49
Aspergillus fumigatus	b	45
Candida albicans		65
Blastomyces dermatiditis		65
Helminths		
Schistosoma mansoni		50

* See also Horwitz, p. 154.

a. Definitive identification of IFNγ as the activating factor is still awaited, although in the case of *R. prowazekii*, this has been provided for fibroblasts (15).

b. Enhancement of killing mainly manifest as reversal of the suppression caused by corticosteroids.

c. An intracellular parasite of erythrocytes and hepatocytes but not macrophages, killed during contact of parasitized erythrocytes with macrophages.

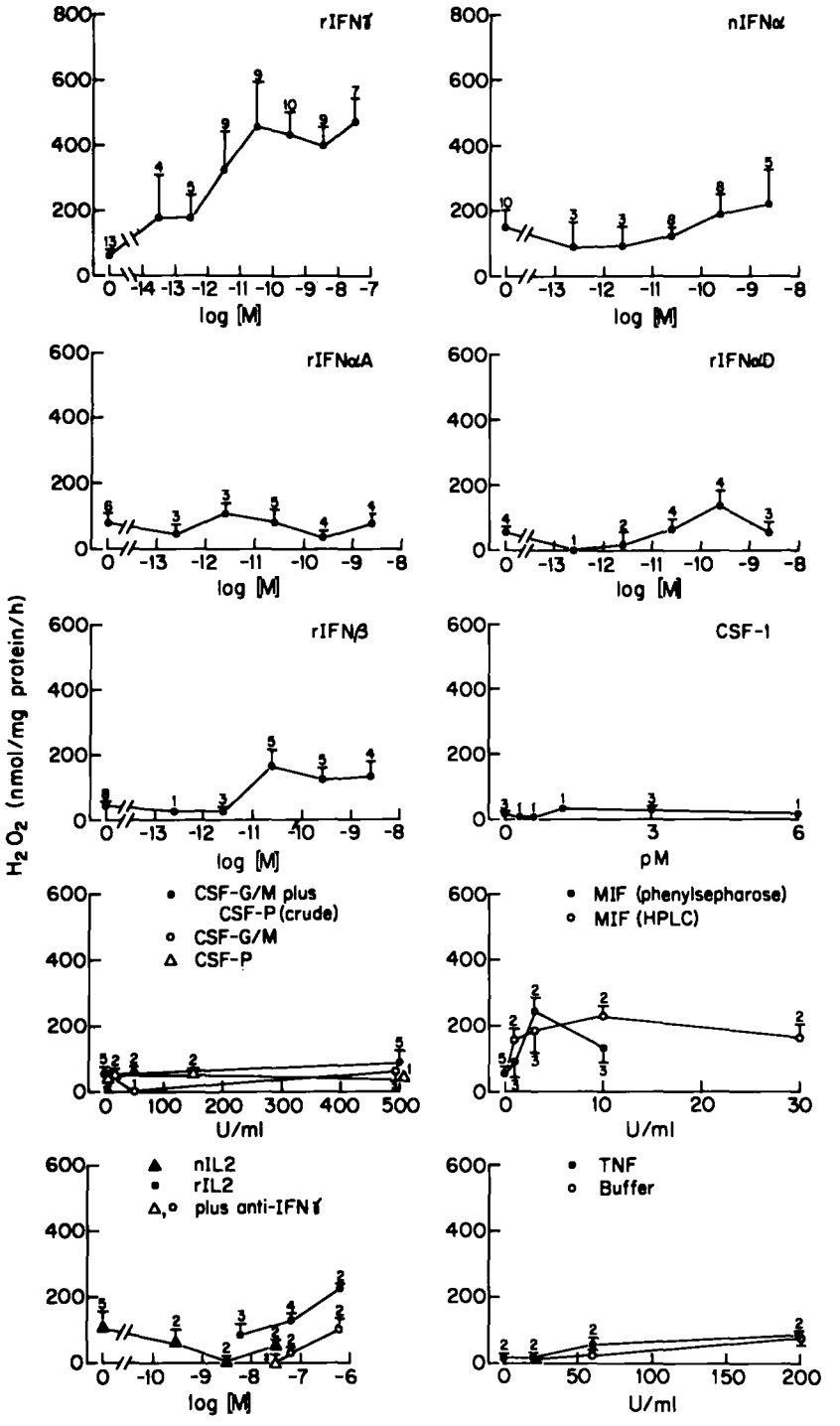
It is frequently debated whether IFNγ can act alone as MAF or whether it requires bacterial lipopolysaccharide (LPS). Most likely, this depends on the experimental system and a key distinction should probably be drawn

between extracellular cytolysis (especially of tumor cells) and intracellular cytotoxicity (toward microbes). Thus, in assays of spontaneous cytotoxicity of macrophages toward tumor cells, in which agents that stimulate the macrophage respiratory burst are not added (such as antitumor antibody), LPS appears to be required (51). Interferon-gamma may have a dual role of enchancing the capacity of the macrophage to produce the cytotoxic factor, and increasing LPS receptors of the cell (52). In response to LPS, the cytotoxic factor may then be released through secretion, shedding, or quite likely, autolysis of the macrophage (52). The mediator of this type of cytotoxicity appears to be TNF (53, Urban et al., manuscript submitted for publication), whose release from macrophages is well known to be regulated by LPS. However, there is as yet no definitive evidence for mediation of macrophage antimicrobial activity by TNF. In contrast, when the experiments involve ingestion of a microorganism, the binding of the microbe itself to the macrophage is often sufficient to trigger a respiratory burst. In my own laboratory, this has been observed with unopsonized *Listeria monocytogenes, Corynebacterium parvum,* bacillus Calmette-Guerín, *Mycobacterium leprae, Toxoplasma gondii, Leishmania donovani,* and *Trypanosoma cruzi.* In this situation, macrophage activation may only require priming of the capacity of the macrophage to mount a respiratory burst (or to manifest oxygen-independent antimicrobial mechanisms). A second signal to trigger metabolic processes may not be needed, beyond what is delivered by the microbe itself. Thus, a requirement for an extraneous second signal such as LPS may not be readily demonstrable. Indeed, no such requirement was found (31), although biologically active traces of LPS in components of the system other than the rIFNγ itself may have been present at levels below the limits of detection of the limulus amebocyte lysate assay. Finally, in some laboratories, LPS itself can substitute for IFNγ in enhancing the respiratory burst capacity of the macrophage (54).

In summary, the evidence in the sections above would appear to argue that IFNγ is both necessary and sufficient to activate macrophages in terms of enhanced ability to kill intracellular microbial pathogens and of its biochemical correlate, enhanced capacity to secrete reactive oxygen intermediates.

Among Cytokines with Effects on Macrophages, Macrophage-activating Factor Activity Is Relatively Restricted to Interferon-Gamma

The conclusion stated above is valid only insofar as it applies to the lymphocyte-conditioned media tested and the conditions under which these media were used. As already noted, there may be cytokines with MAF activity not present in these media in active concentrations or whose


rIFNγ
nIFNα
rIFNαA
rIFNαD
rIFNβ
CSF-1
CSF-G/M plus CSF-P (crude)
CSF-G/M
CSF-P
MIF (phenylsepharose)
MIF (HPLC)
nIL2
rIL2
plus anti-IFNγ
TNF
Buffer
log [M]
pM
U/ml
H2O2 (nmol/mg protein/h)

biologic activity requires other experimental conditions to be manifest. For example, neither IFNα, IFNβ, nor colony-stimulating factor-1 (CSF-1) are likely to be present in media conditioned by lymphocytes.

To address this issue, we attempted to test individually and over a wide range of concentrations, each of the available cytokines known to have effects on macrophages. As shown in Figure 2, rIFNγ and macrophage migration inhibitory factor (MIF) augmented human macrophage H_2O_2 releasing capacity, while pure or highly purified preparations of native IFNα, rIFNαA, rIFNαD, rIFNβ, CSF-1, CSF-G/M, pluripotent CSF, interleukin 2 (IL-2), and B lymphoma-derived TNF lacked this effect. When induction of antitoxoplasma activity was studied in parallel, only rIFNγ was effective (55). The partial effects seen with MIF were variably affected by monoclonal antibodies to MIF and to IFNγ and thus require further investigation. In a murine system, enhancement of the ability of macrophages to secrete reactive oxygen intermediates was induced by CSF-1 (56), an effect not observed in my laboratory. The basis for this discrepancy is not known.

Recently, Ryotaro Yoshida and I have reexamined the effects of IFNα and IFNβ on macrophages. If human monocytes are maintained in culture for longer periods of time than in the earlier studies (31), they develop responsiveness to IFNs α and β in terms of enhanced H_2O_2-releasing capacity. At the same time, complex interactions develop between these IFNs on the one hand, and IFNγ on the other. These will be described elsewhere (manuscript in preparation).

Thus, MAF activity is not a property common to many cytokines that interact with macrophages, but is highly restricted to a few of them. Among the already-identified products of antigen-stimulated T cells, IFNγ is the only one so far shown to act as MAF.

Recombinant Interferon-Gamma Activates Macrophages In Vivo: Studies with Tissue Macrophages in Mice

From the above work, the possibility remains that macrophage activation by IFNγ could be an artifact demonstrable only during in vitro cul-

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Figure 2. Hydrogen peroxide release from phorbol myristate acetate-challenged human macrophages after 3 days incubation in the indicated cytokines. Means + standard error of the mean for the number of experiments noted above each point, each with cells from a different donor. Abbreviations: nIFNα, natural interferon-alpha; CSF-G/M, colony-stimulating factor for granulocytes and macrophages; CSF-P, colony-stimulating factor pluripotent; HPLC, high pressure liquid chromatography; nIL2, natural interleukin 2. Reprinted from Nathan et al. (55).

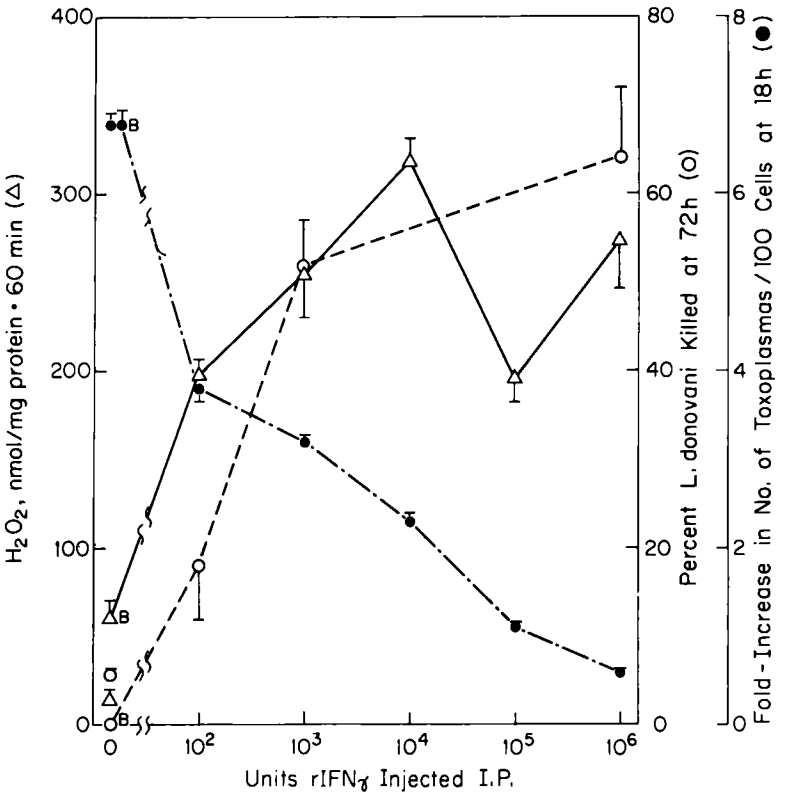

Figure 3. Effect of injection of rIFNγ of mouse origin on the capacity of mouse peritoneal macrophages to release H_2O_2 (Δ), inhibit the replication of *T. gondii* (●), and kill *L. donovani* (O). Mice were injected intraperitoneally (I.P.) with the indicated nominal number of antiviral units of rIFNγ (purified from transfected *E. coli* and provided by Genentech, Inc.) 18 hours before collection of peritoneal cells. The actual amount of rIFNγ injected was less than the nominal amount, because of loss by adsorption to the syringe. Points marked "B" are results for mice injected with diluent alone. The other data for 0 units of rIFNγ are from uninjected mice. Reprinted from Murray et al. (33).

tivation. Conditions in vivo, such as degradation of IFNγ or inhibition of its actions by other stimuli, might block its MAF activity.

In fact, rIFNγ proved highly effective in activating mouse peritoneal macrophages after its injection intraperitoneally (Figure 3), intramuscularly, or intravenously (33). It appeared that resident tissue macrophages underwent activation, because there was no evidence for recruitment of new cells into the peritoneal cavity. The total number and differential count of peritoneal cells was unchanged and monocytes and granulocytes were not increased. Extremely low doses of rIFNγ were effective (Figure 3).

In addition to the above results with *Toxoplasma* and *Leishmania*, in which macrophage activation was assessed in vitro after administration of rIFNγ in vivo, other studies have addressed the fate of rIFNγ-treated mice during infection with organisms that primarily parasitize macrophages: *Listeria monocytogenes* (42), *Toxoplasma gondii* (57), and *Mycobacterium intracellulare* (44). In each case, rIFNγ inhibited the course of the infection.

Recently, recombinant IL-2 (rIL-2) was also reported to protect mice from toxoplasmosis (58). In that study, injection of the peritoneal cavity with rIL-2 before and after injection of parasites into the same site may have accelerated the onset of local cell-mediated immunity and resultant IFNγ-dependent macrophage activation. The authors directly examined macrophage activation only at a late time point, when macrophages were already activated in the controls (mice given toxoplasmas without rIL-2), and the kinetics of the onset of macrophage activation were apparently not considered. It would be interesting to test the effect of monoclonal antibody to IFNγ on the protection afforded by rIL-2 in this system (see below).

Monoclonal Antibody to Interferon-Gamma Prevents Recovery of Mice from Listeriosis

The first clearcut evidence for a physiologic role of endogenous IFNγ in recovery from a bacterial infection comes from experiments in which monoclonal antibody to IFNγ led to marked increases in the number of *Listeria monocytogenes* in the organs of infected mice. Many of the antibody-treated mice died, whereas control animals eliminated the infection and survived (59). The mechanism of action of the antibody in this setting was not proved, but it was shown that macrophages from the control animals exhibited signs of activation not demonstrable after injection of the antibody. To rule out nonspecific effects of the monoclonal antibody, it would be of interest to show that rIFNγ could reverse its effects. It will also be important to conduct similar experiments in a variety of infectious diseases in animals. Needless to say, for ethical reasons, monoclonal antibody to IFNγ cannot be tested in human subjects with infections.

Interferon-Gamma Can Activate Mononuclear Phagocytes in Humans

The challenge remained, whether macrophage activation by IFNγ was demonstrable in vivo not only in mice, but also in humans. The first opportunity to address this came in conjunction with phase I trials of rIFNγ in cancer patients. This was not an optimal setting for two reasons. First, it was not ethical to take serial samples of tissue macrophages from such patients. Thus, only blood monocytes were available for study, and

they already have features seen in tissue macrophages after activation, such as a capacity to release large amounts of reactive oxygen intermediates and to kill efficiently a variety of microorganisms. Only small further increases in these properties could be expected when working with monocytes. Second, the studies were chemotherapeutically inspired and intentionally employed the highest doses of rIFNγ the patients could tolerate, whereas macrophage activation, if it is a physiologic function of IFNγ, should be optimal at low concentrations. Despite these limitations, it was critical to know whether the effects of rIFNγ on macrophages in vitro would be preserved upon its injection in humans.

Indeed, 11 of 13 patients studied responded to rIFNγ with clearcut enhancement of H_2O_2-releasing capacity in their monocytes (Figure 4) (60).

Actions of Recombinant Interferon-Gamma in Human Subjects with Defective Cell-mediated Immunity

There is no evidence that patients with malignancies have a deficiency of endogenous IFNγ. Their monocytes seemed to be activated by exogenous rIFNγ, but only huge doses were tested and only blood monocytes were studied. The issue remains, whether low doses of rIFNγ would reverse features of disease associated with deficiency of endogenous IFNγ and with failure of tissue macrophages to be activated. Such a demonstration may be the final step necessary to conclude that IFNγ is a physiologic MAF in humans.

Such a demonstration is now underway in lepromatous leprosy (Nathan et al., manuscript in preparation). In this disease, exuberant growth of *Mycobacterium leprae* within macrophages marks their activation as inadequate, in striking contrast to the paucibacillary macrophages of tuberculoid leprosy. There may be no intrinsic defect in the ability of macrophages of lepromatous individuals to respond to MAF, because in vitro such cells are indistinguishable from those of normal individuals in their oxidative response to rIFNγ (Kaplan et al., manuscript in preparation). Instead, the lymphocytes of lepromatous subjects may generate inadequate amounts of MAF at sites of infection with *M. leprae*. Thus, in contrast to tuberculoid patients, lepromatous subjects are anergic upon skin testing with *M. leprae* and mobilize few lymphocytes in their lesions, especially cells bearing helper markers (61). The keratinocytes overlying lepromatous lesions are negative when stained for HLA-DR antigens, in contrast to those above tuberculoid lesions (see Cohn, p. 285). Since rIFNγ regulates keratinocyte HLA-DR expression in vitro (62), this observation is consistent with a deficiency of endogenous IFNγ in lepromatous lesions. Finally, exposure of lepromatous patients' blood lym-

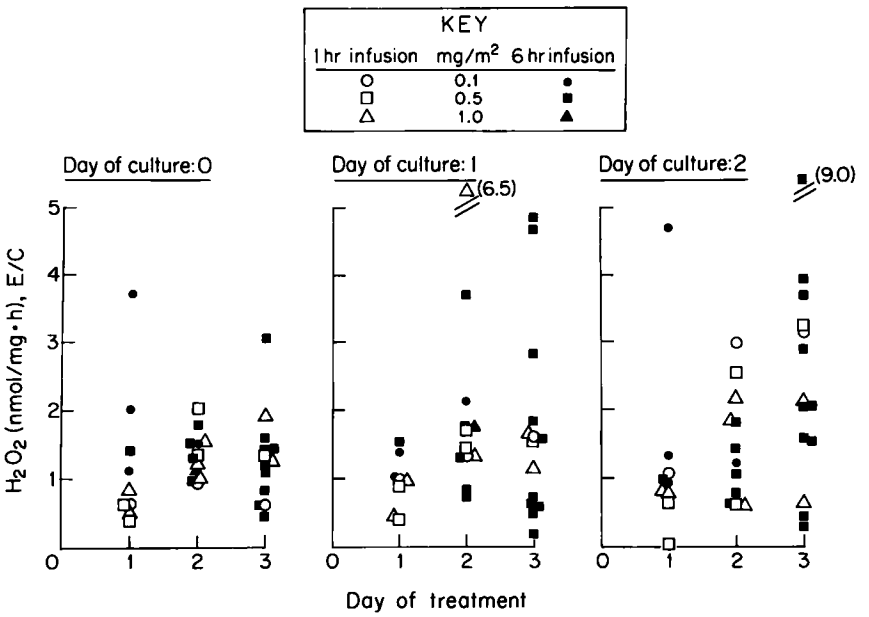

Figure 4. Effect of infusion of rIFNγ on H_2O_2 secretory capacity of monocytes from each of 13 subjects with advanced malignancies. Postinfusion (experimental, *E*) values are expressed as the fold increase (>1) or decrease (<1) relative to preinfusion (control, *C*) values obtained with the same subject's monocytes cultured for the same period of time prior to assay in the presence of phorbol myristate acetate. Day 1 of treatment refers to cells collected immediately after the first infusion. Day 2 of treatment refers to cells collected the next day before any further treatment. Day 3 of treatment refers to cells collected on the third day before that day's treatment. With 1-hour infusions (alternate day schedule), only one treatment was given during the period shown. With 6-hour infusions (daily schedule), two treatments were given during this period: on day 1 and on day 2. All recipients of 1-hour infusions showed unchanged or suppressed responses in monocytes collected immediately after the infusion (open symbols at day 1 of treatment), associated with a marked decrease in typical monocytes recovered from the blood at that time. In addition, two individuals receiving 6-hour infusions were nonresponsive on repeated testing, accounting for 14 of the 17 solid symbols below the line of unity. The overwhelming majority of other postinfusion tests showed increases. Overall, 11 subjects showed increases in at least 67% of tests conducted at any time greater than 1 hour after the start of infusion.

phocytes to *M. leprae* in vitro fails to elicit release of MAF (63) or IFNγ (see Cohn, p. 285).

We therefore asked whether rIFNγ could convert some features of lepromatous lesions toward the tuberculoid pole, which in the natural setting is associated with a more favorable and often self-limited clinical course, and which has been a goal of earlier trials of immunotherapy.

Because there was no prior experience with a recombinant product of the immune system in a nonviral infectious disease in man, and because leprosy patients are prone to immunologically mediated exacerbations, we chose a conservative approach: intralesional injection of rIFNγ in extremely low doses.

As will be described elsewhere (Nathan et al., manuscript in preparation), intradermal injection of 1 or 10 μg of rIFNγ daily for 3 days induced local induration, infiltration by monocytes and T cells, proliferation of keratinocytes, decrease in epidermal Langerhans cells, and intense display of Ia antigens on keratinocytes as well as on cells of the dermal infiltrate. These are all features of delayed-type hypersensitivity reactions. In addition, there were limited observations consistent with, but not proving, a reduction in the number of bacteria in macrophages in the injected sites.

These observations, added to the other evidence reviewed above, suggest that IFNγ released from lymphocytes may mediate many of the manifestations of delayed-type hypersensitivity and cell-mediated immunity.

Prospectus

Recombinant interferon-gamma is now poised for clinical studies in an area where few imagined it might have a role: attempted induction of macrophage antimicrobial activity in individuals with inadequate cell-mediated immunity. Targets might include lepromatous leprosy, leishmaniasis, tuberculosis, and atypical mycobacteriosis. In addition, there may be a rationale for experimentation with rIFNγ in infectious diseases where macrophages are not the principal infected cell, such as malaria and Chagas' disease. This hardly will close the book on IFNγ. On the contrary, it fuels further interest in a molecular understanding of the mechanisms of antimicrobial activity elicited by IFNγ and their place in cell-mediated immunity in its broad perspective.

Summary

This chapter has reviewed the evidence for the identity of the principal lymphocyte product that activates macrophages. For the purpose of this review, macrophage activation is defined as the enhancement, by immunologically specific reactions, of the nonspecifically expressed antimicrobial function of macrophages, and of its close biochemical correlate, the capacity to secrete reactive oxygen intermediates. Media conditioned by clonally unselected lymphocyte populations that contain MAF also contain IFNγ.

Four independently derived monoclonal antibodies against IFNγ in mice and humans remove all detectable MAF from such supernatants. Macrophage-activating factor copurifies with IFNγ from these media. Pure, recombinant IFNγ (rIFNγ) has MAF activity in vitro. The ability of rIFNγ to enhance macrophage antimicrobial activity has been demonstrated with at least 15 different pathogens, including bacteria, protozoa, fungi, and helminths. This is a relatively restricted activity among cytokines with known effects on macrophages. Among T cell products that have already been biochemically characterized, MAF activity so far seems to be a property of IFNγ alone. The potency of rIFNγ as MAF is greater than as an antiviral or antiproliferative agent, suggesting that MAF activity may be a physiologic function of the molecule. In support of this hypothesis, injection of low doses of rIFNγ activates mouse macrophages in vivo and improves the ability of the murine host to handle a variety of experimental infections. Conversely, monoclonal antibody to rIFNγ prevents the spontaneous resolution of infection with at least one facultative intracellular bacterium in mice. In humans, the first studies of rIFNγ were limited to cancer patients receiving relatively large doses. This resulted in enhanced reactive oxygen intermediates production by blood monocytes. Recent studies have employed much lower doses of rIFNγ in patients with lepromatous leprosy, in whom there is a defect in the ability of T cells to produce IFNγ in response to the infecting organism. In these individuals, intralesional rIFNγ appears to induce a variety of changes characteristic of the reactions of delayed-type hypersensitivity and cell-mediated immunity. Thus, under the conditions studied, IFNγ appears to be both necessary and sufficient for activation of macrophages by lymphocytes. Moreover, the role of IFNγ as MAF appears to be a physiologic one.

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Destruction of Tumor Cells by Mononuclear Phagocytes: Models for Analyzing Effector Mechanisms and Regulation of Macrophage Activation

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Introduction

The concept of macrophage activation dates to Élie Metchnikoff (1), who noted that macrophages from immune animals have perfected their powers for chemotaxis and for the digestion of microbes. During the 1920s and 1930s, many workers, including Alexis Carrel (2) and Warren and Margaret Lewis (3) observed that these cells underwent profound changes in morphology during prolonged culture in vitro. Max Lurie (4) in the 1940s found that host resistance to tuberculosis depended on macrophages, and that macrophages from immune animals had enhanced microbicidal properties (4). Studies on macrophage activation culminated in the 1960s in the elegant studies of George Mackaness (5), Robert North (6), and their colleagues, who dissected the phenomenon of cellular resistance to facultative intracellular parasites such as *Listeria monocytogenes*. They showed convincingly that cooperation between specifically sensitized T cells and the local accumulation of large, angry macrophages, which Mackaness subsequently termed activated, was necessary for destruction of the bacteria. At the same time, Zanvil Cohn (7) clearly elucidated the fundamental biochemical changes that accompany the development of mononuclear phagocytes in culture. The story of macrophage activation took a dramatic turn in the early 1970s, when Peter Alexander and Bob Evans (8) and then Jack Remington and John Hibbs (9) observed that macrophages activated for microbicidal destruction could also selectively and efficiently destroy tumor cells without the aid of antibodies. From these studies and others, the concept arose that macrophages can develop into an activated state in which they are capable of destroying intracellular microbes and

extracellular tumor cells (for reviews, see References 5, 6, 10, and 11). During the century from 1880 to 1980, work from many laboratories thus amplified, extended, and yet fundamentally confirmed the basic concept of Metchnikoff that macrophages are vital cells in host defense, and that their ability to perform such defensive functions can be enhanced by previous exposure to the intruding agent.

During the past two decades, the host-wide system of mononuclear phagocytes has been found to fulfill a wide variety of other roles in the host's economy (12, 13). Starting with the observaton that macrophages are potent secretory cells (14), macrophages are now established to participate in many important host mediator and defense systems including the complement cascade, the coagulation response, and the acute inflammatory response (12, 15, 16). Furthermore, these cells, by virtue of both soluble mediators and cell–cell interactions are important to regulation of the immune system, the proliferaton of other cells, and hematopoiesis (12, 17, 18). Finally, macrophages are important in homeostasis, inasmuch as they regulate the organismic metabolism of a variety of molecules including lipids and iron (19,20).

These two broad themes of macrophage function have proven to be closely intertwined, for almost all functions of macrophages depend upon their state of development (12). Because macrophages respond to a wide variety of inductive and suppressive signals, their competence to perform these multiple functions can be either enhanced or diminished. Given that macrophages possess over 50 receptors on their surface and secrete over 80 defined molecular constitutents (12, 15), it should not be surprising that these physiologic/biochemical properties are independently regulated, and thus that induction of competence for individual functions is independently expressed. The emerging picture of the importance and complexity of macrophage development raises some questions of interest: (*a*) How does one define macrophage activation? (*b*) What is the basic model for development of macrophage functional competence (i.e., is competence for multiple functions acquired concomitantly, progressively, or disparately?) (*c*) What extracellular signals regulate the development of macrophages? and (*d*) How are extracellular regulatory signals transduced in the cytoplasm to effect functional changes? In this chapter we describe studies from this laboratory, in which we have used the analysis of macrophage cytolytic function to address some of these questions.

Models of the Destruction of Tumor Cells by Activated Macrophages

Macrophage-mediated Tumor Cytotoxicity

Macrophages, when obtained from sites of infection with a chronic intracellular parasite such as bacillus Calmette-Guerin, efficiently lyse tumor cells in vitro (for review, see Reference 21). The lytic process, which takes place over several days, is independent of antibody, contact-dependent, and remarkable in that the macrophages distinguish between neoplastically transformed cells and their untransformed counterparts (21). There is an absolute requirement in such lysis for activation of the macrophages, because other macrophages cannot mediate the destruction of tumor cells in this circumstance. For the past five years, we have dissected this form of lysis extensively in our laboratory and have been able to divide this complex process into two fundamental components—target binding and secretion of lytic mediators (for reviews, see References 21–23) (Table I).

The first step in macrophage-mediated tumor cytotoxicity (MTC) is the selective recognition by activated macrophages of tumor cells (21–23). This is manifested by active adherence between the two cells, such that they become firmly attached or bound to one another. For example, when nonadherent tumor cells are added to a culture of activated macrophages, up to one-half of the tumor cells become strongly bound to the macrophages. Such binding can be inhibited by plasma membranes from a variety of tumor cells, though not by membranes from untransformed cells. The establishment of binding is itself complex, in that it is an active process on the part of the macrophages. Several lines of evidence have been adduced that the capture may be mediated by a receptor on the macrophages (22). We have recently been able to dissect the overall establishment of capture into several stages and further quantify the avidity of cell-cell binding in each stage (24).

Activated macrophages, though not unactivated macrophages, secrete a potent lytic substance which, in the absence of serum, selectively lyses tumor cells (21–23). Although the active principle has not been purified to homogeneity, several lines of evidence suggest that it is a serine protease of approximately 38,000 mol wt (25). Of interest, protease inhibitors, which block the lytic and proteolytic activities of the isolated material, inhibit the lysis of tumor cells by activated macrophages (21–23). It remains to be established whether the cytolytic principle directly causes destruction of tumor cells or cooperates with other molecules to produce the final toxic substance(s) that effect target injury.

Table I. Three Basic Forms of Tumoricidal Activity by Macrophages

	MTC	*Fast ADCC*	*Slow ADCC*
Two stages of lysis	Yes	Yes	Yes
Contact dependent	Yes	Yes	Yes
Triggering necessary	Yes	Yes	Yes
Recognition system	Tumor cell		
	Binding site	$Fc_{\gamma 2a}R$, $Fc_{\gamma 2b/\gamma 1}R$. $Fc_{\gamma 3}R$	$Fc_{\gamma 2a}R$, $Fc_{\gamma 2b/\gamma 1}R$, $Fc_{\gamma 3}R$
Mediators of kill (in relative order)	Cytolytic protease; others possible.	H_2O_2O; others?	H_2O_2 or other ROI; others?
Stage where regulated			
Target binding	Yes	No	No
Secretion of mediators	Yes	Yes	Yes
Time required for kill (*hours*)	16–48	4–6	16–48
Inductive signals	IFNγ, LPS	MAF, IFNγ	IFNα
Suppressive signals	α_2-Macroglobulin-protease complexes	?	IFNγ, LPS

Data have been adapted from Adams and Hamilton (12). Abbreviations: ADCC, antibody-dependent cellar cytotoxicity; IFN, interferon; LPS, lipopolysaccharide; MAF, macrophage-activating factor; MTC, macrophage-mediated tumor cytotoxicity; ROI, reactive oxygen intermediates.

The overall lytic process in MTC depends upon a close interrelationship between binding and release of the cytolytic material (21–23). Multiple lines of evidence have indicated that macrophages first selectively bind and hold tumor cells at their surface and then secrete the cytolytic principle and other toxic substances as well to effect kill (21–23). Binding appears to serve several roles that include recognition of the target for destruction, provision of a protected space between macrophages and targets, and enhanced and more rapid secretion of the cytolytic principle. The capacity to bind tumor cells depends upon response to macrophage-activating factor (MAF) or interferon gamma (IFNγ), whereas the capacity to secrete cytolytic activity follows exposure to bac-

terial lipopolysaccharide (LPS) or alternative second signals (21–23). Just one of the lines of evidence supporting this model of activation and execution of lysis has been the analysis of inbred strains of mice (26). Mice of the A/J strain, which respond poorly to lymphokines, acquire the ability to secrete the cytolytic principle but not to bind targets; alternatively, mice of the C3H/HeJ strain, which are unresponsive to endotoxin, acquire competence for target binding effectively but not for secretion of the cytolytic principle. Of interest, cytolysis can be restored in both of these circumstances either by providing artificial binding targets via a lectin bridge or via stimulation of secretion of the cytolytic material by an alternate signal (27; Marino, P. A., and D. O. Adams, unpublished observations).

Antibody-dependent Cellular Cytotoxicity

Macrophges have long been recognized to effect a rapid antibody-dependent cellular cytotoxicity (ADCC) interaction with antibody-coated red cells (for reviews, see references, 28, 29). Several years ago, it was noted that macrophages activated in vivo by BCG rapidly lysed a variety of nonadherent, lymphoid tumor cells coated with specific antisera over 4–6 hours (28, 29). Other macrophages are not so capable. Although there is a close degree of correlation between the respective abilities for MTC and ADCC during a period of several hours, competence for the two functions is not invariably associated, and the two can be distinguished (28). Lysis in the rapid ADCC can be divided into two steps: (*a*) an initial binding of antibody-coated targets occurring for 10–20 minutes and independent of temperature and (*b*) a lytic step occurring for 3–6 hours and dependent on incubation at 37°C (30). In a series of insightful studies, Nathan has provided compelling evidence that active oxygen intermediates are a major lytic mechanism (29), although the potential role of other lytic mediators remains to be defined (31).

The lysis of tumor cells by macrophages in the presence of antibodies, however, is often not so rapid. Many laboratories have found that macrophages can lyse tumor cells in the presence of antibody for 1–2 days in culture (32, 33). Tumor cells lysed in this circumstance represent a broad range of targets including adherent and nonadherent cells, sarcomas, carcinomas, lymphomas, and melanomas.

We have recently attempted to analyze this slow lytic reaction in some detail (Johnson, W. J., et al., manuscript submitted for publication). Of particular interest, the macrophages that mediate slow ADCC are quite distinct from those that mediate rapid ADCC. Specifically, macrophages activated for mediating the slow form of ADCC represent selected subsets of macrophages elicited by sterile inflammatory agents; BCG-activated

macrophages actually have a diminished capacity (see Table II). Like the rapid ADCC by macrophages, the slow form of kill is divisible into an initial, rapid, temperature-independent binding step and a second, slower lytic step. The lytic step can be inhibited by approximately 60% with either anaerobiosis or glucose deprivation (which inhibits the respiratory burst) and by sodium thioglycollate, a potent but nonspecific scavenger of hydrogen peroxide (Johnson, W. J., et al., manuscript submitted for publication). At present, the data suggest that this type of ADCC reaction may be mediated in part by reactive oxygen intermediates, and that a role for other toxic mediators remains to be established.

The role of which isotype or isotypes of antibody are most effective in ADCC has been a particularly interesting problem (33). Clearly, antibodies of the IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 isotypes can mediate an ADCC response by macrophages to either erythrocytes or tumor cells (for review, see Reference 33). The relative potency of these isotypes, however, remains to be established, because they are usually directed against different epitopes on the tumor cells. The recent introduction of isotypic switch variants of monoclonal antibodies (MAbs) directed against the same epitope has proven a useful tool in analysis of this problem (34). Our results with such MAbs indicate that all of the four preceeding isotypes are effective (Johnson, W. J., et al., manuscript submitted for publication) but that antibodies of the IgG_{2a} isotype are the most effective (Adams, D. O., unpublished observation). Of interest, antibodies of this isotype are more effective with BCG macrophages in mediating the rapid ADCC with selected targets and with responsive macrophages elicited

Table II. Competence of Various Macrophages to Mediate Three Types of Tumor Cell Lysis

	Resident Mϕ	*Responsive Mϕ*	*Primed Mϕ*	*Activated Mϕ*
MTC	–	–	–	+ + + +
Fast ADCC	–	–	–	+ + + +
Slow ADCC	–	+ + + + or –	+ + + + or –	–

Data have been adapted from Adams and Hamilton (12). Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; MTC, macrophage-mediated tumor cytotoxicity; Mϕ, macrophage.

by thioglycollate broth in mediating slow ADCC of other targets (Adams, D. O., unpublished observation).

How can one reconcile these somewhat disparate observations? We interpret the data now available that the rapid and slow types of ADCC are but variations on the same fundamental biological process. A critical variable determining whether lysis is rapid or slow may be the relative susceptibility of targets to lysis by reactive oxygen intermediates (ROI). In examining an extremely limited panel of targets, we have found that two targets susceptible to rapid ADCC are relatively susceptible to lysis by reagent hydrogren peroxide (i.e., ED_{50} approximately 5×10^{-5} M) but that targets susceptible to slow lysis are more resistant (i.e., ED_{50} greater than 5×10^{-3} M H_2O_2) (Adams, D. O., unpublished observation). In light of the well-known capacity for BCG-activated macrophages to mount an effective and rapid respiratory burst (12), one might hypothesize that some ROI-sensitive targets are susceptible to lysis by a rapid respiratory burst whereas other ROI-resistant targets require prolonged contact with effector cells (prolonged release of ROI, release of other toxic mediators, or both?). At present, we are investigating these questions.

Signals Regulating Competence for Destruction of Tumor Cells

Competence for completing MTC as being acquired in a sequence of operationally defined stages has been well documented from the work of Hibbs, Meltzer, and Russell (35–37). In brief, young mononuclear phagocytes taken from sites of inflammation respond well to lymphokines and hence are termed responsive macrophages; resident peritoneal macrophages by contrast respond poorly to lymphokines (Table III). Neither will become cytolytic upon addition of relatively small amounts of endotoxin (i.e., 10 ng/ml). After the responsive macrophages have been treated with lymphokine for 6–8 hours, they become receptive to the inductive influences of small amounts of LPS and are termed primed macrophages. The primed macrophages, when given low amounts of endotoxin, achieve full lytic competence for mediating MTC. Thus, a lymphokine such as IFNγ and second signals such as LPS, high concentrations of lymphokine, heat-killed *Listeria monocytogenes*, muramyl dipeptide, and malyelated/acetylated proteins, strictly regulate the activation of murine macrophages (12, 38, 39). The two must be given in a defined order and previous exposure of the macrophages to lymphokine markedly lowers the amount of LPS (or other second signals) that must be subsequently given to induce lytic competence.

In order to define precisely the state of activation of a given population of macrophages and to be able to analyze signals regulating activa-

Table III. Stages of Activation of Macrophage-mediated Tumor Cytotoxicity by Murine Peritoneal Macrophages

	Resident Mϕ	*Responsive Mϕ*	*Primed Mϕ*	*Activated Mϕ*
Source in vivo	Unmanipulated peritoneal cavity	Peritoneal cavity injected with sterile inflammatory agent	Peritoneal cavity injected with pyran copolymer (MVE-2)	Peritoneal cavity injected with *Propionibacterium acnes* or BCG
Source in vitro	Recently explanted peritoneal Mϕ	Resident peritoneal Mϕ cultured for several days	Responsive Mϕ cultured with lymphokine or interferon gamma	Primed Mϕ cultured with second signal (bacterial endotoxin)
Operational definition for MTC	Do not respond to lymphokine and endotoxin by becoming cytolytic	Respond to lymphokine followed by endotoxin by becoming cytolytic	Respond to endotoxin by becoming cytolytic	Fully cytolytic

Data have been adapted from Adams and Hamilton (12). Abbreviations: BCG, Calmetti-Guerin bacillus; MTC, macrophage-mediated tumor cytotoxicity; MVE-2, Mϕ, macrophage.

tion more precisely, we have developed a panel of quantitative objective markers expressed by macrophages in the operational stages of development (Table IV) (40). These stages can be used on macrophages undergoing development in vitro or in vivo (41, 42). Responsive macrophages have many characteristics of the well-described inflammatory macrophage (10). Competence for selective binding of tumor cells is induced by lymphokine alone so that macrophages, whether primed in vivo or in vitro, are capable of effecting such binding. Competence for release of the cytolytic principle, by contrast, is regulated in a more complex fashion. Activated macrophages spontaneously secrete small amounts of cytolytic principle and secrete much larger amounts more rapidly after selective binding of tumor cells (42). Primed macrophages do not secrete the cytotoxic principle at all until challenged with LPS, whereas responsive macrophages do not secrete the principle either spontaneously or upon challenge with LPS. Thus, the requirement for two signals given in defined order is partially explained by how these signals regulate the capacities for completion of MTC. We have gone on to develop a larger panel of additional biochemical, physiologic, and antigenic markers that characterize macrophages in the primed, responsive, and activated stages of development (Table IV). These markers have proven to be applicable to macrophages obtained in vitro and in vivo and reliably predict the stage of macrophage development (43).

The signals that regulate competence for ADCC in vitro are not yet so well defined. The Fc receptors of macrophages can be up- and down-regulated in a complex fashion by several signals including lymphokines (44, 45). Furthermore, lymphokines are known to induce competence for the oxidative burst during several days of culture (46). We have recently observed that macrophages treated with IFNγ followed by LPS lose competence for the Fc receptor-mediated respiratory burst and concomitantly lose competence for mediating the slow variant of ADCC (47).

The utility of markers in discriminating among populations of macrophages activated in various ways has recently shed information on the role of macrophages in the destruction of tumors in vivo. Tumors undergoing immune-mediated attack can contain macrophages activated for MTC (48, 49). We have analyzed two models of tumors undergoing immune-mediated attack initiated by monoclonal antibodies of the IgG_{2a} isotype directed against the tumor. In both models, tumors from the treated animals are destroyed and the animals live. Control tumors treated with a monoclonal of the IgG_{2a} isotype, which does not react with the tumor cells, a monoclonal of the IgG_{2b} isotype, which reacts with the tumor cells, or with nothing continue to grow progressively until death of the

Table IV. Markers of the Stages in the Activation of Macrophage-mediated Tumor Cytotoxicity

	Resident Mϕ	*Responsive Mϕ*	*Primed Mϕ*	*Activated Mϕ*
Expression of mannose receptor	±	+ + + +	+	+
Expression of transferrin receptor	±	+ + + +	+	+
Content 5′ nucleotidase	+ + +	+	+	+
Capacity for selective binding of tumor cells	−	−	+ + + +	+ + + +
Prepared to secrete cytolytic protease	−	−	+ + + +	+ + + +
Secrete cytolytic protease	−	−	−	+ + + +
Kill tumor cells in MTC	−	−	−	+ + + +
Phagocytosis of EIgG	+	+ + +	+ + +	+ + +
Phagocytosis of EIgMC′	−	+ + +	+ + +	+ + +
Secretion of neutral proteases	−	+ + + +	+ + + +	+ + +
Expression of Ia molecules	−	−	+ + + +	+ + + +
Expression of LFA-1 Molecules	−	−	+ + + +	+ + + +

Data have been adapted from Adams and Hamilton (12). Abbreviations: LFA-1, lymphocyte function-associated antigen; MTC, macrophage-mediated tumor cytotoxicity; Mϕ, macrophage.

animal (50; Johnson, W. J., et al., manuscript submitted for publication). In both models, we have found two striking changes in the tumors: (*a*) the number of macrophages has been increased from two- to two and one-half-fold; (*b*) macrophages within the regressor, although not the progressor tumors, have been activated for the slow form of ADCC. Of note, macrophages in the regressor tumors have not been activated for either of the rapid variant of ADCC or for MTC. These data are consistent with other observations from the literature that the number of macrophages within a tumor undergoing antibody-mediated attack may be an important limiting variable in determining the size of the tumor that can be destroyed (for references, see 50 and Johnson, W. J., et al., manuscript submitted for publication). These observations suggest that further emphasis should be placed on analyzing the populations of leukocytes of all types, including macrophages, within tumors undergoing rejection in regard to both number and function.

Molecular Basis of Regulation of Activation

Overview

Interferon gamma and LPS represent two important signals for inducing competence for MTC and regulating both rapid and slow ADCC (12, 47, 51). These two potent signals also regulate a host of other important macrophage functions (Table V). Of note, the two signals can act in several ways: in cooperation as described for MTC, independently, in parallel, or antagonistically. Yet, little is known about the molecular basis of how these signals affect macrophage function. Recently, a receptor for IFNγ has been described on murine macrophages (52). The fundamental basis of the effects of LPS on cells remain to be established, although current models center upon a two-step reaction in which the endotoxin is adsorbed onto the cell surface and subsequently is intercalated into the lipid bilayer (53, 54). The subsequent basis of how these signals are transduced in the cytoplasm to effect changes in function is poorly understood.

Biochemical Effects of Interferon Gamma and Lipopolysaccharide

We have recently begun to ask what cytoplasmic second messenger signal systems are initiated by the interaction of responsive and primed macrophages with, respectively, IFNγ and second signals such as LPS. We have found that IFNγ induces pertubatons in intracellular levels of Ca^{++} over 10–15 minutes and also enhances the *potential* function of protein kinase C (PKC) (55; Somers, S. D., manuscript submitted for publication). Lipopolysaccharide initiates phosphorylation of a defined set of proteins, a

Table V. Examples of Various Effects of Interferon Gamma and Lipopolysaccharide on Macrophages

Effect type	*Signals(s)*	*Actual result*
Parallel	LPS or IFNγ	↑ LFA-1 molecules
	LPS or IFNγ	↓ Transferrin receptor
Independent	IFNγ	↑ Competence for respiratory burst
	LPS	↑ Acid phosphatase
Antagonistic	IFNγ	↑ Ia molecules
	LPS	↓ Ia molecules
Cooperative	IFNγ plus LPS	↑ $\text{Competence}_{\text{MTC}}$
		↓ Membrane fluidity
		↓ Respiratory burst to Fc complexes
		↓ Competence for slow ADCC

Data have been drawn from Adams and Hamilton (12). Abbreviations as in preceding tables.

transient wave of synthesis of a defined set of proteins, and expression of new mRNA for the cellular proto-oncogenes c-fos and c-myc (55a, 55b; Introna, M., et al., manuscripts submitted for publication). The pattern of enhanced expression of phosphoproteins closely resembles that induced by phorbol myristate acetate (PMA), a direct stimulant of PKC (55a, 56). Peptide mapping by limited proteolysis of three of the major phosphoproteins demonstrate striking similarity if not actual identity among the phosphate containing peptides stimulated by either LPS or PMA, suggesting the substrates are identical and are phosphorylated by the same protein kinase activity (55b). Thus, the data imply that endotoxin induces protein phosphorylation via PKC. The newly synthesized proteins are of interest because they appear within 1 hour after application of LPS and disappear rapidly after that, despite continued application of LPS (the half-lives of some of the proteins are 1–3 hours as determined by pulse chase experiments) (55b). Interferon gamma regulates both of these effects, in that prior treatment of cells with IFNγ enhances the mag-

nitude of protein phosphorylation and reduces the amount of LPS required to initiate protein synthesis (55a, 55b).

Role of Biochemical Events in Activation

We have also addressed the question of whether these biochemical events are important to activation by attempting pharmacologic mimicry of tumoricidal activity (Somers, S. D., et al., manuscript submitted for publication) (Table VI). Responsive macrophages, when treted with 10^{-8} M PMA plus 10^{-7} M calcium ionophore (A23187), enter the primed state of development and can be triggered to lysis by LPS. The effects of IFNγ in down-regulating the receptor for transferrin and in inducing surface expression of Ia molecules can also be mimicked by these agents (57, 58). We have further used inbred mice for analysis of this question. Interferon gamma does not enhance the potential of PKC in A/J mice, but priming of macrophages for cytolysis can be induced in these mice readily by treatment with A23187 and PMA (a potent and direct stimulant of PKC) (Hamilton, T. A., unpublished observation). When macrophages from C3H/HeJ mice are treated with LPS, enhanced protein phosphorylation is not observed. The application of an alternate second signal, such as heat-killed *L. monocytogenes*, restores both expression of cytolysis and enhanced protein phosphorylation. Taken together, this evidence implies that the biochemical events we have described may not only accompany macrophage activation but may well be integral to its successful completion.

A Biochemical Model of Macrophage Activation

These biochemical data can be placed together in an integrated model of macrophage development (Figure 1). As can be seen, we currently believe that we have identified four elements that may be critical to macrophage activation: pertubations in intracellular levels of calcium, expression of cellular oncogenes, protein phosphorylation via potentiated PKC, and synthesis of new proteins. A minimal interpretation of this model is that IFN_γ prepares the cell for subsequent signaling events by raising intracellular calcium levels and the *potential* of PKC, whereas LPS initiates a wave of protein phosphorylation via PKC, expression of certain cellular oncogenes, and synthesis of new proteins. These beginning studies only hint at the complexity of other events that undoubtedly take place in activation. For example: What immediate second messages are initiated by IFNγ and by LPS? What is the identity of the phosphoproteins and of the new proteins? What relationship do these events bear to the synthesis of surface proteins such as LFA-1 and to each other?

Table VI. Lines of Evidence Implicating Protein Phosphorylation and Ca^{++} Fluxes in Activation

Effects of activating signals on intracellular biochemical changes	
IFNγ	Elevates intracellular Ca^{++} and modulates *potential* activity of PKC
LPS/*L. monocytogenes*	Enhance protein phosphorylation (via PKC?); stimulate synthesis of new proteins
Pharmacologic mimicry of activation	
PMA and/or A23187	Reproduce effects of IFNγ, including modulation of cell surface protein expression and priming for tumor cytolysis
Analysis of genetic deficiencies	
A/J mice	Deficient response of IFNγ, in terms of expression of cell priming for tumor cytolysis, surface protein expression (except LFA-1), and modulation of PKC
C3H/HeJ mice	Deficient response to LPS, in terms of activation for tumor cytolysis, enhanced protein phosphorylation, and new protein synthesis
Restoration of genetic deficiencies	
A/J Mice	PMA + A23187 leads to enhanced phosphorylation of proteins, fluxes of Ca^{++}, and restoration of priming for lysis
C3H/HeJ Mice	*L. monocytogenes* initiates enhanced phosphorylation of proteins, synthesis of new proteins, and activation for lysis

Data have been taken from Hamilton et al. (55), Weiel et al. (55a), Hamilton et al. (55b), Weiel et al. (57), and Strassmann et al. (58) and Somers et al., manuscripts submitted for publication. Abbreviations as in preceding tables; PKC, protein kinase C; PMA, phorbol myristate acetate.

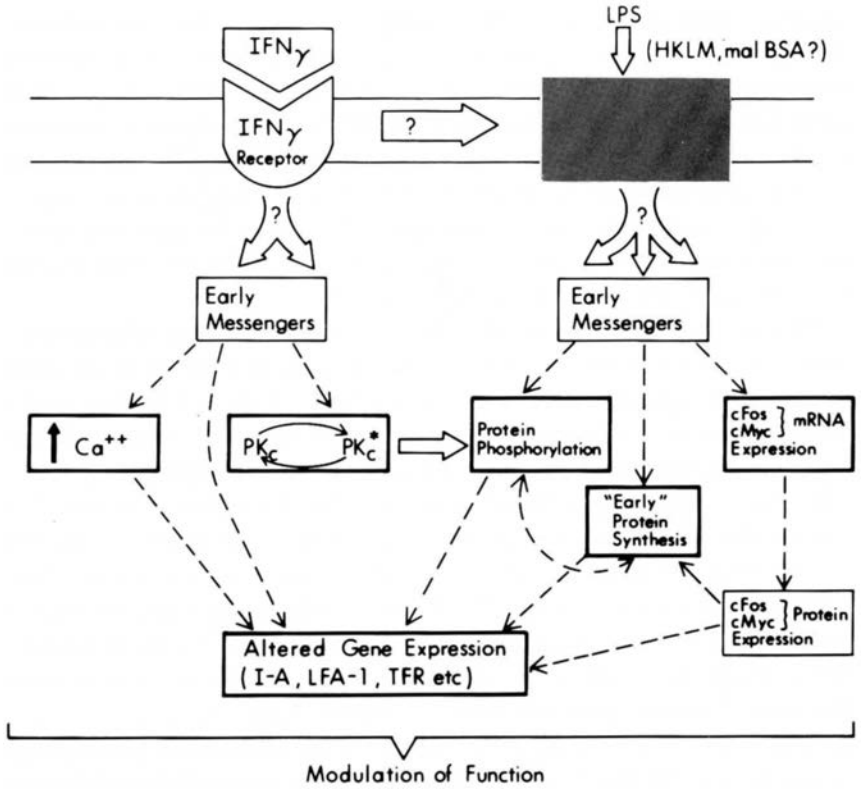

Figure 1. A model of signal transduction in macrophage activation. Boxes with heavy borders are *established* elements; boxes with light borders are *postulated* elements. Dashed lines indicate *potential* interrelationships. Data from Hamilton et al. (55), Weiel et al. (55a), Hamilton et al. (55b) Weiel et al. (57), and Strassmann et al. (58) and Somers et al., Introna et al., manuscripts submitted for publication.

Discussion

Based on our analyses of macrophage acquisition for competence to destroy tumor cells, we have proposed a fundamental definition of macrophage activation (12, 21). *Macrophage activation* is acquisition of competence to perform a complex function such as MTC, rapid ADCC, slow ADCC, destruction of microorganisms, or regulation of secondary T cell immune response (12, 21). The ability to perform such a complex function is subserved by expression of the appropriate physiologic and biochemical properties, such as competence to bind tumor cells, ability to secrete hydrogen peroxide, number/affinity of Fc receptors, content of a given enzyme, etc. (12, 21). In other words, such physiologic and biochemical capacities

usually represent the full cellular expression of separate and independently regulated gene products, whereas competence for a function represents the integrated result of operative interactions between these products. For example, activation for MTC has been shown to comprise (*a*) the capacity for selective binding of tumor cells and (*b*) the capacity for release of cytolytic principle (21–23). By extension then, the signals inducing activation for a given function are the sum of the inductive signals inducing the requisite capacities specific for that function. This general concept has been verified for MTC (21–23).

We have further suggested that there are multiple forms of activation, when one so defines activaton (12). Our general concept of the development of macrophages of the tissues is depicted in Figure 2. In this model, young mononuclear phagocytes come into the tissues (12). If, on the one hand, strong inductive signals are not present, the macrophages are generally down-regulated, perhaps to protect the host from unwanted or undesirable effects of macrophages with enhanced physiologic capacities or functions, such as the ability to secrete reactive oxygen intermediate or present antigen to T cells (12). If, on the othe hand, inductive signals such as inflammatory mediators or specific products of T cells are present, the macrophages undergo further physiologic and biochemical maturation and ultimately gain functional activation. Depending on the precise mix of inductive and suppressive signals, various forms of macrophage activation may be observed. Consideration of the various forms of destruc-

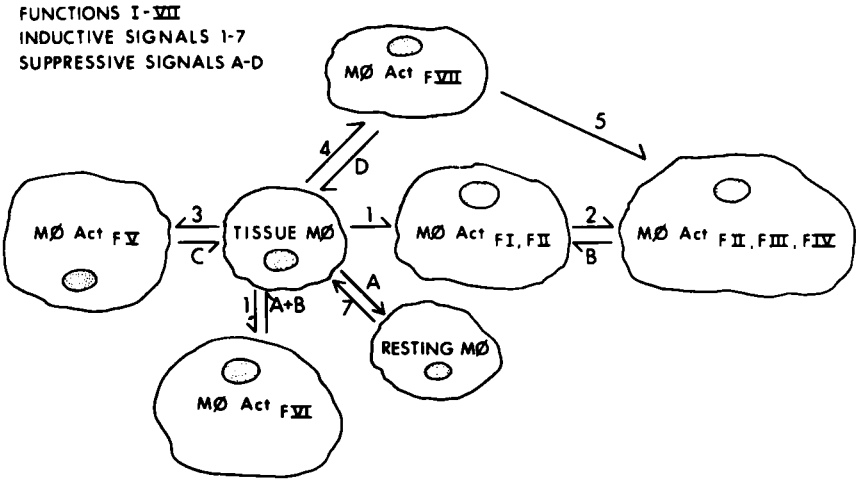

Figure 2. A model of macrophage development in the tissues from Adams and Hamilton (12) (with permission).

tion of tumor cells generally provides support for this model (Table II). Too, recent studies analyzing activation for microbicidal function point to disparate paths of activation within the general category of activation for destructive purposes (59). If one considers competence for other complex functions, emerging evidence would thus seem to support this general concept of pleuripotent development of macrophage competence. From a teleologic point of view, it would be neither efficient nor wholly beneficial for the macrophage to express fully all 50 surface receptors and release all 80 secretory products at the same time. Though much remains to be learned about the multiple forms of activaton, the specific inductive and suppressive signals that can impinge upon macrophages, and the physiologic and biochemical capacities that support each of these forms of activation will undoubtedly emerge over the coming years. The general approach employed to date in addressing the problem has been a reductionist one: first, isolate a specific form of altered competence; second, dissect the mechanism(s) of the altered function, in order to define the requisite physiologic and biochemical capacities; and third, isolate the specific inductive and suppressive signals that regulate these capacities.

We have now entered an exciting new era in the analysis of macrophage activation—analysis of how macrophage activation is regulated at the molecular level. From the limited insights we have already gained into this problem, it is clear that macrophage development of functional competence is complexly regulated at the intracellular level by interplay between multiple signal transduction signals. As we gain further insights into the key mechanisms and the balances that operate between inductive and suppressive signals, we anticipate a deeper understanding of how acquisition of macrophage functional competence is actually regulated. Such studies not only offer the potential for a useful model of acquisition of function by normal cells but may lead to the development of specific pharmacologic compounds that can modulate macrophage activation. For example, the knowledge that PKC is an important regulator of cellular development and differentiation has recently led to the synthesis of a potent series of water-soluble analogues of PMA (60). We have recently found that such compounds induce the activation of macrophages for tumoricidal function (Somers, S. D., et al., manuscript submitted for publication). In summary, further exciting questions and challenges in the analysis of macrophage activation lie ahead in the coming decade for those working on this interesting problem of cellular development and regulation.

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The Granule Exocytosis Mechanism for Lymphocyte Cytotoxicity

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The ability of lymphocytes to inflict lethal damage on other cells has been recognized for over 20 years, and the "killer lymphocytes" responsible for this activity have received considerable study because of their possible relationship to the rejection of allografts and tumors. Because cytotoxicity can be quanitatively measured in vitro by using a rapid and relatively simple assay, it is the best-defined, direct lymphocyte-mediated effector function of the immune response. Nevertheless, the detailed mechanistic process by which killer lymphocytes cause the death of their target cells has remained controversial and somewhat mysterious. Over the past several years, evidence favoring a mechanism, which we call the granule exocytosis model, has accumulated to the point where we now feel that this can largely account for the known properties of the several classes of lymphocyte cytotoxicity. This chapter describes this model in terms of previous work on cytotoxicity mediated by cytotoxic T lymphocytes (CTL) and large granular lymphocytes (LGL). It also summarizes recent findings that lend further support to this model, emphasizing experiments from our laboratory. Because an extensive review of most of the relevant literature was recently published (1), readers are referred there for most citations to the original literature. Only other reviews and the most recent original publications are referred to here.

Figure 1 shows a diagrammatic view of the granule exocytosis model for lymphocyte cytotoxicity, which can be broken down into five steps. These are discussed in detail below.

The first step is the binding of the target cell by the effector. A specific binding of target cells that reflects the cytotoxic specificity can be demonstrated for both CTL and LGL under defined conditions; it is most easily studied by counting killer-target pairs in the microscope (2), which has recently been extended to the cell sorter (3–5). This binding is mediated by receptors on the killer cell that bind to ligands on the target cell. Cytotoxic T lymphocytes utilize the increasingly well-defined T cell antigen receptor, which recognizes a target membrane complex that includes major histocompatibility comples (MHC) antigen. Large granular lymphocytes

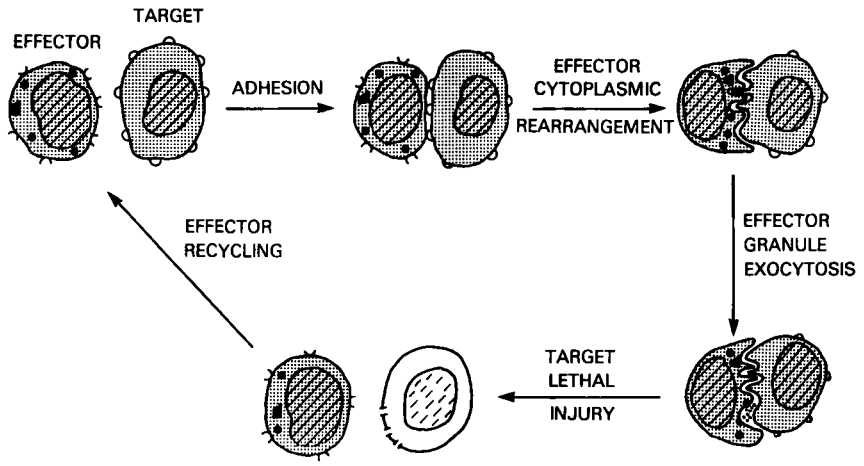

Figure 1. The granule exocytosis model for lymphocyte-mediated cytotoxicity, showing individual steps of the cytotoxic process described in detail in the text.

have two different receptors that mediate target cell binding and killing. One is an Fc receptor which recognizes IgG–antigen complexes on target cells to trigger antibody-dependent cell-mediated cytotoxicity (ADCC), and the other is an undefined class of natural killer (NK) receptors which recognize poorly defined moieties on some tumor cells, various immature hemapoietic cells, and probably some microorganisms to give rise to the "natural killing" phenomenon. It should not be imagined that the specific adhesion observed between killer lymphocytes and their targets is merely a molecular interaction between the effector cell receptors and target ligands. This specific binding requires magnesium (calcium is a poor substitute), and is inhibited by agents that disrupt the cytoskeleton. Cytotoxic T lymphocyte–target adhesion, but not that of LGL, is inhibited by low temperature and energy metabolism blockers.

In addition to the specific receptors, other membrane proteins on the effector cells appear to aid in target cell adhesion, as detected by the ability of monoclonal antibodies against effector cell antigens to block both target cell lysis and specific adhesion. The lymphocyte function-associated (LFA) antigens, especially LFA-1, appear to interact with target cell ligands to "reinforce" the specific interaction, in both the CTL and NK lytic processes (6). Lymphocyte function-associated antigen 1 also plays a role in other functionally important lymphocyte–lymphocyte interactions. The CTL–target interaction can be enhanced by the Lyt 2/T8 molecules in the case of MHC class 1-restricted recognition (7), and by the L3T4 molecules for MHC class 2-restricted recognition (8). The need for such

enhancement appears less if the specific receptor–target antigen interactions are strong. However, killer–target adhesions per se do not insure target cell lysis, because not all specific killer–target pairs show target lysis after prolonged incubation, and many conditions of incubation will yield good binding of CTL and LGL to non-target cells that are not detectably lysed.

The second step in the killing process is termed effector cell cytoplasmic rearrangement. The specific membrane receptors of the killer cell are postulated to trigger intracellular second messengers, which are presumably similar to those triggered by other receptor–ligand interactions. These induce an asymmetry in the killer cell with respect to its bound target, thus allowing a directed secretory process to occur in the next step. Morphologic observations have demonstrated such asymmetry of the effector cell in killer–target pairs prior to observable target cell damage. Electron microscope studies of CTL and NK killing have revealed a preferential localization of killer cell lysosomal granules and the Golgi complex towards the bound target cell. Further evidence for such asymmetry has been obtained using fluorescent antibody localization of the Golgi complex and of the cytoskeletal proteins actin and tubulin. These changes appear to require calcium (9), as might be expected from recent studies showing a rise in cytoplasmic calcium triggered by the T cell receptor (10). Studies of both CTL (11) and NK (12) lytic processes have defined a "lethal hit" or "programming for lysis" stage of the cytotoxic process defined by its requirement for calcium and independence of magnesium. This stage follows the binding stage, and in the above scheme both the cytoplasmic rearrangement step and granule exocytosis would appear to be included within the programming-for-lysis stage as originally defined.

According to the granule exocytosis model, this cytoplasmic rearrangement occurs in order to create a specialized junctional region between the killer and its bound target, and to allow a directed exocytosis of killer cell secretory granules into this region. This junction and the granule exocytosis can be considered as analogous to a neuronal synapse and the controlled secretion of neurotransmitters that occurs there.

Not all the granules need to move to the side of the killer cell adjacent to the bound target, because it is not expected that even a majority of the granules will undergo exocytosis when killing a single target cell. A major function of the specialized junction between the two cells is to create a restricted volume for diffusion of the secreted product, thus achieving a high local concentration at the target membrane even if only a few granules undergo exocytosis. It seems likely that the killer cells save some

and probably most of their ammunition for future encounters, as expected from the observations that both CTL and NK cells are capable of killing several targets within a few hours.

The third and most crucial step in the scheme of the lymphocyte cytotoxic mechanism is granule exocytosis. This implies that killer lymphocytes must contain cytoplasmic granules, which in the case of LGL are illustrated in Figure 2. Granules of LGL in other species can have different electron-microscopic (EM) morphologies, but distinct granules can be observed by light microscopy. Both ADCC and NK activities are associated with the granule-bearing lymphocyte populations in humans, rats, and mice. For CTL, however, the presence of these granules was not widely appreciated even though most EM studies of CTL–target conjugates had pointed out their existence. For both CTL and LGL, the number of granules per cell is far fewer than for granulocytes or mast cells. In both cases the granules are several hundred nanometers in diameter, and are positive for lysosomal enzymes by EM histochemistry. A functional role for granules in cytotoxicity is clearly implied by EM studies of CTL- and NK–target pairs, where it was observed that granule lysosomal enzymes, or osmophilic material like that in effector cell cytoplasmic granules, are deposited in the region where the two cells are opposed.

Among the possible mechanisms for target cell damage inflicted by killer lymphocytes, membrane damage had long attracted attention. Evidence for the formation of large pores in red cell ghosts attacked by LGL in ADCC came from a sieving behavior of trapped marker release. Surprisingly, markers up to 14 nm in diameter were released from such targets, but not larger markers. Complement attack on the same ghosts gave a similar pattern but a 5-nm diameter marker cutoff, implying smaller pores. The demonstration of porelike structures with a 15-nm internal diameter on ghost membranes attacked by the lymphocytes suggested that cytotoxic lymphocytes may act by releasing a complementlike protein, which inserts into bilayers and forms a larger pore. The EM observations of both NK and CTL killing shows that the porelike structures originate in the cytoplasmic granules of the killer cells, and this has been confirmed by study of the contents of the granules, discussed below. Thus this EM evidence shows that granule exocytosis does occur, and that material in the granules becomes associated with target membranes during the lytic process. Details of the nature of the material in the granules will be discussed below. As the biochemical nature of the contents of killer cell granules becomes better defined, it will be possible to quantitate the exocytosis event by following the release of individual components, and studies of this type are currently underway.

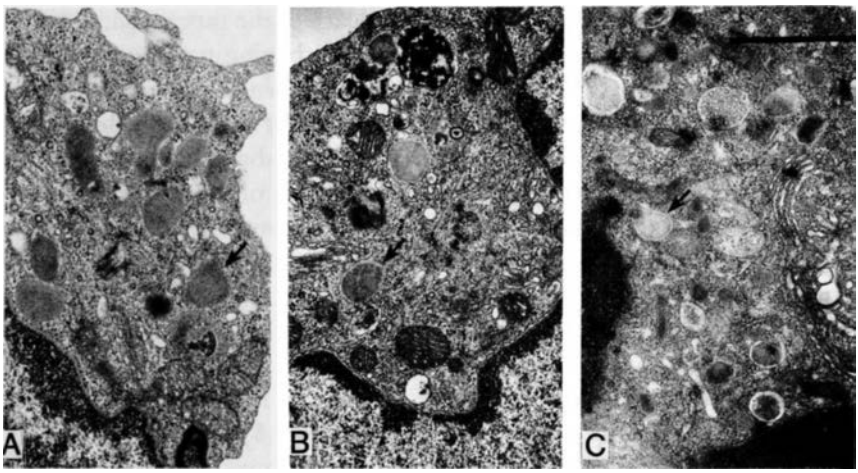

Figure 2. Cytoplasmic granules of rat large granular lymphocytes and rat NK tumor cells. Electron-microscope images of sections of cytoplasm near the nuclear indentation of rat peripheral blood LGL (A) and rat LGL tumor cells (B and C). The characteristic cytoplasmic granules (*arrows*) appear as grainy gray staining round organelles surrounded by a limiting membrane. The close association of the granules with the Golgi complex is seen in all three cases, and a centriole is clearly seen in panel A. Bar, .62 μm. From Millard et al. (13).

The fourth step in the scheme of the mechanism is the lethal injury to the target cell. The creation of a membrane pore is strongly implicated from the evidence discussed above, and such pores could kill the cell by direct leakage of cytoplasmic contents in the case of the large pores demonstrated in the ghosts, or by colloid osmotic lysis resulting from the equilibration of the small ions across the membrane. It is known from earlier studies of both CTL and NK mechanisms that killer–target contact is not required for some time prior to target cell death as measured by ^{51}Cr release—the "killer cell-independent" phase of the process.

Although the formation of a pore in the target membrane would appear to be the principal component of the lethal injury, other granule components may also play a role. It has been shown that some nucleated cells can repair membrane pores induced by complement, and it is possible that such repair processes could protect cells against the cytolysin pores. However, such pores may allow the penetration into the target cells of other toxic granule components, possibly including nucleases, other granule enzymes, and various lymphotoxins. These could inflict the damage which cause the actual death of the target cell or the destruction of internal viruses.

The last step in the lytic process is the death of the target cell, detachment of the killer cells, and the potential for the beginning another killing event. Studies of the CTL lytic process have shown that there is a killer cell-independent phase of the process that does not require divalent ions, is relatively temperature-independent, and has a half time of about 1.7 hours (14). The most straightforward prediction of the granule exocytosis model and the known properties of the granule cytolysins would be that the target cell would die within a few minutes after granule exocytosis, inasmuch as nucleated cells die rapidly after addition of the isolated granule cytolysins to target cells. The more prolonged time for the killer cell-independent phase of the CTL lytic process has not been explained. For NK killing, a similar step in the lytic process has been described, with a half time of several minutes (15).

After the lethal hit has been delivered, the killer cell leaves the dying target and is capable of killing other target cells by the same cycle. Little is known of the mechanism of detachment of the killer cell from its injured target. The ability of the killer cell to encounter another target cell is dependent on its motility, which time-lapse experiments have documented as a clear feature of cytotoxic lymphocytes.

Components of Cytotoxic Lymphocyte Granules

From the proposed mechanism of lymphocyte cytotoxicity just described, it is clear that granule exocytosis is the critical step. The properties of lymphocyte granule components are therefore crucial to an understanding of this lytic mechanism. Granules from various types of cytotoxic lymphocytes have been isolated in several laboratories, and a number of similar components have been described. The cell sources for granule isolation have been lymphocyte lines cultured in interleukin 2 (IL-2) and the rat LGL tumor lines which have NK and ADCC activity. Questions can be raised as to the relevance of these cells to previous studies on CTL and NK killing, because CTL grown in IL-2 have been shown to acquire NK-like and other "anomalous" killing specificities and thus potentially other lytic mechanisms, and the NK tumor lines show slower killing kinetics than do normal NK cells. However, biochemical studies require substantial numbers of pure cells, and further experimentation will be required to verify the applicability of granule biochemistry studies to the lytic mechanisms of various types of cytotoxic lymphocytes.

My colleagues and I have concentrated on the granules from rat LGL tumor cells. To purify these granules (15), we have ruptured the cells by nitrogen cavitation, removed the nuclei by filtration, and sedimented the

homogenate on self-forming Percoll gradients. The granules form a band at the bottom of this gradient, where they are free of other organelles detectable by EM and enzymatic markers. This region of the gradient also is associated with the major peak of the lysosomal enzymes acid phosphatase, β-glucuronidase, β-*N*-acetyl-glucosaminidase, aryl sulfatase, and α-fucosidase. This is expected in view of previously described histochemical evidence for the lysosomal nature of cytoplasmic granules in CTL and LGL. Similar dense granules containing lysosomal enzymes can be purified from some, but not all, other lymphoid cells. Working in our laboratory, Dr. William Munger has recently found that both CTL and the NK tumors contain DNAse activity which is capable of releasing DNA from isolated nuclei in normal media. This activity does not appear attributable to the normal lysosomal DNAse II enzyme. According to the granule exocytosis model, this DNAse would be released during exocytosis and then gain entrance to the target cell by the pores implanted by the granule cytolysin. We think that this mechanism is capable of explaining the rapid DNA release during CTL killing (16). It should be noted, however, that this DNAse is also found in granules prepared from at least one noncytotoxic lymphocyte clone.

The purified granules of LGL tumor cells contain four to five major protein bands seen in reduced SDS gels; these include one to two in the range of 60,000–65,000 mol wt and three bands in region of 30,000 mol wt. Proteins of this general size have also been seen in granules from cloned CTL grown in IL-2 (17, and unpublished work from our lab).

In spite of EM images suggesting that granules contained lipid, phospholipid analysis revealed only minimal amounts, such as would be present in the limiting membrane around the granules. Some carbohydrate was present, including the uronic acids characteristic of proteoglycans.

Cytolysins

Because the major motivation in the study of lymphocyte granules has been in the lytic mechanism of cytotoxic lymphocytes, attention has largely centered on the potent lytic activity displayed by isolated granules. We have termed this activity "cytolysin." As seen in Figure 3, the cytolysin is simply demonstrated by standard lytic assays using a variety of different types of cells as targets (18). The lytic process is rapid, going to completion for both erythrocytes and nucleated cells within a few minutes at room temperature and within 1 minute at 37°C. The role of calcium is critical in the lytic activity of granules, as seen in Figure 4. In the absence of divalent ions, the granule lytic activity is not observed, while calcium

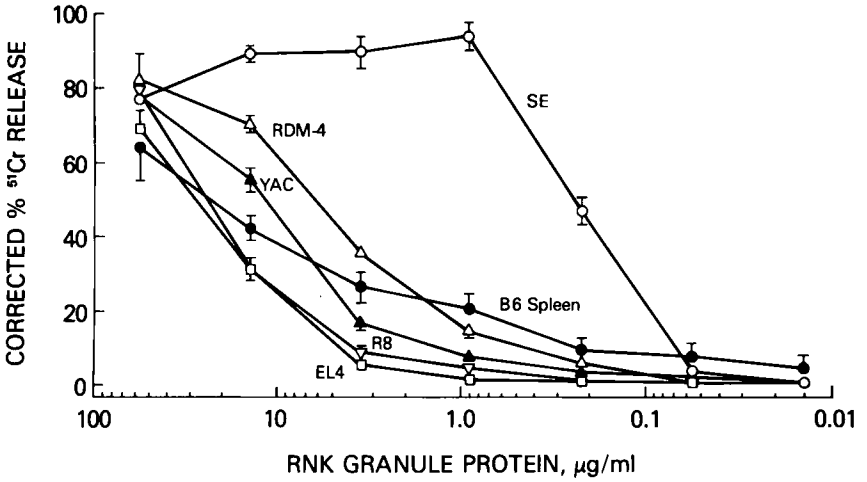

Figure 3. Cytotoxic effects of purified LGL tumor granules. Granules purified by Percoll gradient centrifugation were lysed by freeze-thaw and diluted in phosphate-buffered saline. Labeled target cells were added in an equal volume of calcium containing medium and the supernatants harvested after 20 minutes. The target cells are sheep erythrocytes (SE), normal splenocytes from C57Bl/6 mice (B6 spleen), the mouse lymphomas YAC-1 and EL-4, the T cell leukemia cell line RDM-4, and the pre-B cell leukemia R8. From Henkart et al. (18).

concentrations in the physiologically extracellular range give good activity. Most other divalent ions are unable to replace calcium, but strontium will do so at about 10-fold-higher concentrations. However, exposure of granule preparations to calcium prior to addition of target cells causes an irreversible loss of the lytic activity over the course of ½ hour at room temperature. This can most readily be visualized as calcium causing an activation of the lytic component leading to an unstable active intermediate. In any case, this dual effect of calcium probably explains why previous investigators had not found this lytic activity in cytotoxic lymphocyte extracts.

Cytolysin activity is detectable in dense cytoplasmic granules of cytotoxic T lymphocytes, peripheral blood LGL, LGL tumor cells, and lymphokine-activated killer cells, all of mouse or rat origin. The quantitative level of cytolysin activity is approximately equal in all these cases on a per cell basis. Most other lymphoid cells, including non-LGL normal lymphocytes and the vast majority of lymphoid tumors and cloned T cell lines, do not contain detectable cytolysin in any fraction of their homogenates. However, we have found granule-associated cytolysin in two cloned T cell lines which do not express lytic activity even in the

presence of phytohemagglutinin. As would be expected from the granule exocytosis model, the presence of the lytic apparatus in cytoplasmic granules is not in itself sufficient to endow the cells with lytic capabilities.

Experiments designed to examine the mechanism of the lytic activity of granules from cytotoxic lymphocytes strongly suggest that a granule component inserts into the target membrane and forms pores. Negative-stain EM observations of frozen and thawed granules incubated with calcium-containing medium show that the pore structures described above form on the granule membranes. These structures are not seen if the same preparations are incubated under identical conditions in the absence of calcium. Only a few minutes of incubation are required for these pore structures to form from the granules. Thus, there is a clear and striking correlation between the cytolytic activity of granules and the appearance of these pore structures with respect to requirements for calcium and time.

The lytic activity of both CTL and LGL granules appears to be entirely accounted for by a single-granule protein component termed a cytolysin or perforin. This 65,000–75,000-mol wt protein is a major granule protein and has been purified by several different approaches (e.g., 19). We have started with purified granules from the rat LGL tumor cells, solubilized the activity with 2 M NaCl, and followed this with gel filtration on ACA 54, where the activity elutes as a single peak corresponding to a roughly 60,000-mol wt protein. This material is further purified on a heparin column, where it is eluted with NaCl to give a single-protein species even after silver-stained sodium dodecyl sulfate gels.

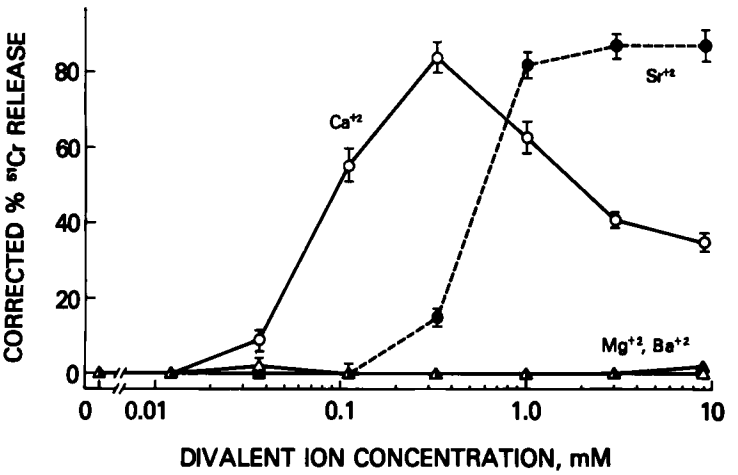

Figure 4. Effect of divalent ions on cytolysin activity of LGL tumor granules. Target cells for this experiment were YAC lymphoma cells. In all cases the divalent ions were added with the target cells, with the final concentration shown. From Henkart et al. (18).

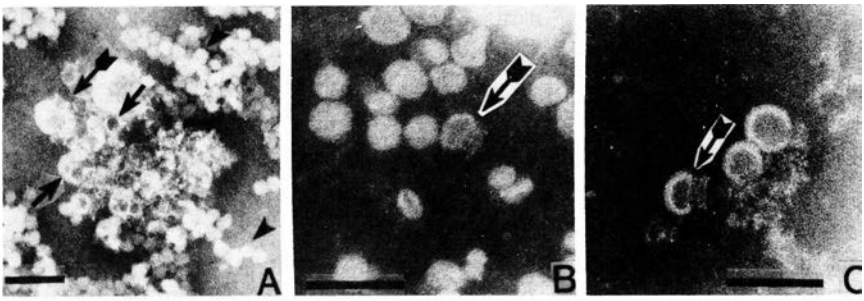

Figure 5. Insertion of cytolysin pore structures into liposomes. Small unilamellar liposomes were permeabilized by LGL tumor cytolysin from granules (A) or from column purified cytolysin (B and C). After carboxyfluorescein release measurements, the preparations were examined by EM after negative staining with phosphotungstate. Pore structures can be seen end-on in panel A (*tailess arrows*), and in side view (*arrows with tails*). In panel A, Percoll particles are visible (*arrowheads*). Bars, 45 nm. From Blumenthal et al. (20).

Because of the pore structures seen in the EM appear similar to those formed by complement and bacterial toxins believed to operate by forming an amphipathic protein complex and insertion into the lipid portion of membranes, we tested the action of the LGL granule cytolysin on liposomes (20). Using the carboxyfluorescein technique to monitor liposome permeability, we observed a dose-dependent and rapid release of this water-soluble marker from both small and large unilamellar liposomes. This process showed the identical divalent ion dependence as was previously described for cell lysis. Variation of the lipid composition of the liposomes had a negligible effect on the marker release, except that liposomes containing synthetic lipids below their transition temperature showed a slower rate of carboxyfluorescein release. Examination of cytolysin-treated liposomes by negative stain EM showed the apparent insertion of the pore structures into the lipid, which was particularly striking for small unilamellar liposomes as seen in Figure 5. Negative-stain penetration into the liposomes was observed when the pore structures were inserted into the lipid, but stain was excluded from others in the same experiment, strongly supporting the simple interpretation of the pore structure being a functional pore.

Further studies on the structure and function of the granule cytolysins of cytotoxic lymphocytes are currently in progress and will undoubtedly shed light on the mechanism by which this water-soluble protein interacts with calcium, inserts into membranes, and aggregates into the large EM-visible structures. From the present data, it would appear that these molecules are one of a class of pore-forming proteins (21), which include

the C9 component of complement, staphylococcal α-toxin, streptolysin O, and perhaps *Escherichia coli* porin.

At the present time there is no direct evidence that the granule cytolysins play a role in the cell-mediated killing process. As previously discussed, there is considerable evidence for a granule exocytosis in both LGL and CTL killing, and the large pores detected by marker release in red cell ghosts are difficult to explain by mechanisms other than the cytolysin. As one approach to obtain further evidence for a role for granule components, we have prepared antibodies to the LGL tumor cytoplasmic granules. These antibodies react with the major protein components of granules on Western blots, and $F(ab')_2$ fragments prepared from them show potent and specific inhibition of the granule cytolysin. These $F(ab')_2$ antibodies specifically and completely inhibit ADCC and NK cytotoxicity by rat peripheral blood LGL without inhibiting the formation of the killer–target adhesions in these systems. These results argue that some component of the granules is necessary for a postbinding step of LGL-mediated cytotoxicity, as predicted by the granule exocytosis mechanism. Further experiments with antibodies against pure cytolysin may directly implicate this protein in the lytic event.

Nevertheless, even without such direct eivdence, the accumulated evidence outlined above is strongly suggestive in the absence of other plausible lytic mediators in the granules.

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Molecular Assemblies in Complement- and Lymphocyte-mediated Cytolysis

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Introduction

The ultimate goal of the immune defense and immune surveillance systems is to eliminate invading cells and organisms that have been recognized as foreign. For this purpose intracellular and extracellular killing systems have evolved that lyse and destroy invading targets.

Extracellular cytolytic activity, defined here as lytic activity independent of phagocytosis, is mediated by humoral and by cellular effector systems. Among the cellular elements cytolytic T lymphocytes (CTL) and natural killer (NK) cells mediate the major part of extracellular killing activity, whereas the humoral compartment is mainly represented by the complement system.

In this chapter the molecular mechanism of target destruction by complement and by cytolytic lymphocytes is described. The reason for considering these traditionally separately discussed systems together comes from recent research developments that indicate some analogies in complement- and lymphocyte-mediated cytolysis.

In Figure 1 the steps leading to target destruction by complement or lymphocytes are outlined in schematic form. The first step in the cytolytic sequence is the specific recognition of foreign targets. Complement utilizes for this purpose component C1 that binds to the Fc portion of antigen–antibody complexes and enzymatically activates the classical pathway. Antibody-independent recognition by complement of highly repetitive molecular structures, such as lipopolysaccharides or carbohydrates, is mediated by C3 and factors B and D in cooperation with factors H and I in a complex reaction sequence.

Recognition by cytolytic lymphocytes is a function of the T cell antigen-major histocompatibility complex (MHC) receptor that confers a degree of antigen specificity similar to antibody-mediated reactions. Not shared by antibody is the property of the T cell receptor to recognize antigen only associated with class I or class II MHC molecules, thus restricting T cells to the recognition of cellular antigens whereas soluble antigens

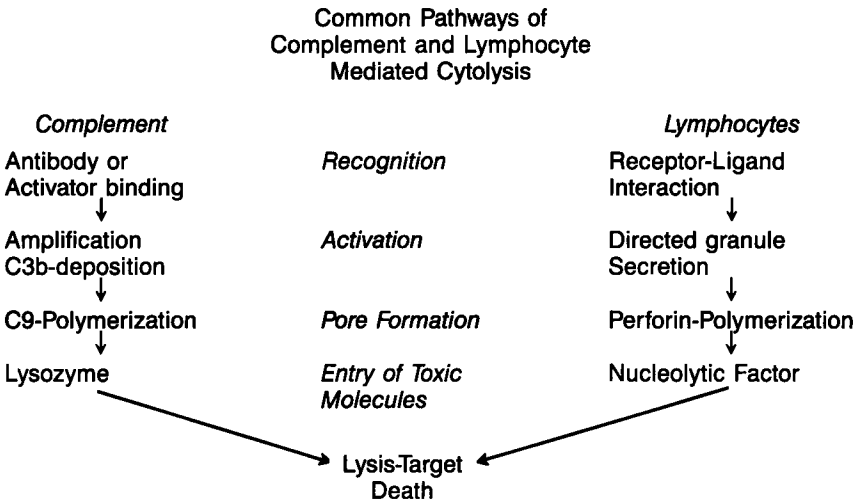

Figure 1. Common pathways of complement- and lymphocyte-mediated cytolysis (for further details see References 1–3, 7, and 12).

are ignored. Adherence to the target subsequent to T cell receptor recognition is enhanced by such additional cell surface molecules as T4 for class II MHC-specific T cell clones, and T8 for class I MHC-specific clones. Lymphocyte function-associated antigen-1 promotes adherence by yet undetermined ligand interaction.

Recognition by NK cells is not MHC restricted. It is possible that NK cells recognize certain structures that are more abundant or altered on certain tumor cells. Carbohydrate, cell surface receptors, and the ligand for T11 have been discussed in this context, however, the precise recognition element(s) is still not clear. It may be speculated that NK cells recognize a variety of structures, in analogy to the alternative pathway of complement, and thus may become activated by different ligands on different cells.

Recognition is followed by activation of the pathway and amplification of the original signal. In complement a relatively complex reaction sequence results in the massive deposition of C3b, which is the prerequisite for the formation of the C5 convertase, the enzyme responsible for the initiation of the assembly of the membrane attack complex. Because complement is a humoral effector system, a complex set of activating, controlling, and inhibiting proteins have evolved that seem to be required to balance rapid activation with excessive complement consumption or premature inhibition. In addition, because all blood cells are continuously exposed to the potentially damaging effects of complement, mem-

brane proteins on erythrocytes, platelets, and leukocytes have evolved that protect homologous cells from attack by complement.

The mode of activation of cytolytic lymphocytes involves the translocation of Ca ions upon membrane receptor–ligand interaction. The subsequent chain of events, which has not yet been studied in detail, results in the directed secretion of cytolytic granules of killer lymphocytes into the interstitial space between killer target conjugates (1, 2).

The actual attack phase of complement and killer lymphocytes shows perhaps the highest degree of homology. Lysis of target cells in both systems is achieved by the membrane insertion of tubular transmembrane complexes arising through the polymerization of monomeric precursor proteins. Transmembrane tubules form large aqueous pores causing cell death by dissipation of transmembrane ionic gradients and the transmembrane potential as well as by causing the loss of macromolecular cellular components. In addition, the transmembrane pores are utilized for the entry of toxic molecules causing internal target cell damage.

This chapter will review the mechanism by which transmembrane tubules are assembled and how they are used by complement and by cytolytic lymphocytes for extracellular killing of target cells. (For more detailed reviews, see also References 1, 3, 7, 11, 12)

Formation of Transmembrane Tubules by Polymerization of Pore-forming Proteins

Complement

Complement component C9, a 73,000-molecular weight (mol wt) hydrophilic glycoprotein, can be induced to polymerize to an amphiphilic, tubular complex of 160Å length and 100Å internal diameter (Figure 2). Polymerizing C9 inserts into phospholipid bilayer membranes, thereby forming stable transmembrane pores with a functional diameter of up to 100Å. Natural membranes are not attacked by polymerizing C9 in the absence of other complement proteins (see below).

C9 polymerization occurs spontaneously a 37°C in a slow reaction; Zn ions accelerate C9 polymerization; the most rapid C9 polymerization is caused by the C5b-8 complex (see below). C9 polymerization is also enhanced in the presence of detergents, chaotropes, or by limited cleavage with tryspin and thrombin.

The polymerization of globular, hydrophilic C9 to the amphiphilic transmembrane tubule of polymerized C9 involves the restricted unfolding of C9 (Figure 3), uncovering hydrophobic domains and neoantigenic sites.

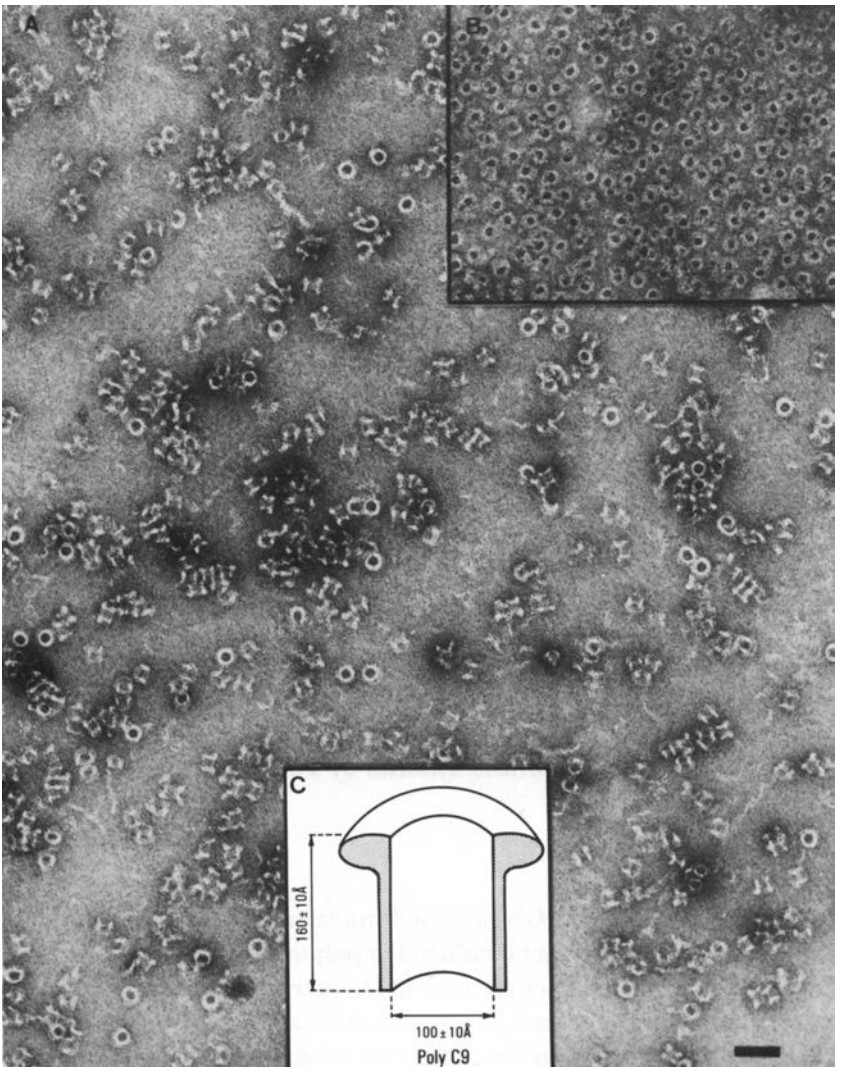

Figure 2. (A) Negative staining electron micrograph of polymerized C9 (poly C9). (B) Complement membrane lesions on cell membranes. (C) Schematic view of poly C9 tubule bisected along its long axis (see also References 3, 6, and 12).

The tubule of polymerized C9 is stable to dissocation by sodium dodecyl sulfate (SDS) and to digestion by proteases. C9 protomers in polymerized C9, in addition, can become disulfide linked by spontaneous disulfide exchange (6). This stabilization of polymerized C9 by tubular polymerization and disulfide linking is important for its resistance against defense

mechanisms of attacked targets (for detailed discussions of poly C9 see also ref 3, 12).

Cytolytic T Lymphocytes and Natural Killer Cells

Cytolytic lymphocytes contain pore-forming proteins (8) designated perforin 1 and perforin 2. Perforin (P1) is a 70,000–75,000-mol wt protein that polymerizes in a Ca-dependent reaction to an amphiphilic, tubular complex of 160Å length and 160Å internal diameter (poly P1). Polymerized perforin 2 (poly P2) consists of a tubular complex of 50–70Å internal diameter (Figure 4). It has been suggested that the subunits of the poly P1 and poly P2 complex are identical.

Perforin 1 is located in the cytoplasmic granules of killer lymphocytes (4, 5). The mechanism of its release and its mode of action are described below. Isolated P1 in the presence of Ca ions attacks membranes without detectable specificity. Erythrocytes and *all* nucleated cells tested as well as synthetic phospholipid membranes can serve as targets. Whether a given target succumbs to poly P1 attack may depend on the activity of repair mechanisms and possibly on the physical nature of the membrane.

Mechanism of Induction of Polymerization of Pore-forming Proteins

Assembly of the Membrane Attack Complex of Complement

The physiologic accelerator of C9 polymerization is the C5b-8 complex. C5b-8 is assembled on target membranes upon proteolytic activation of

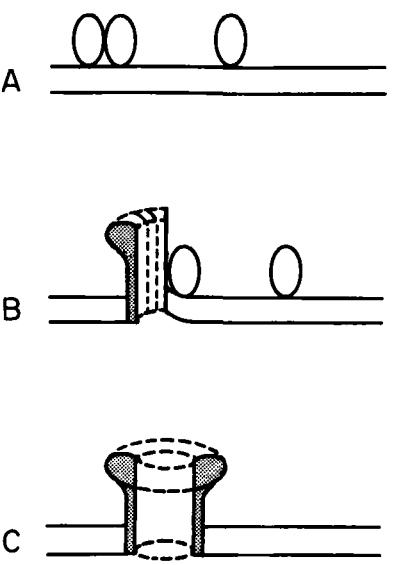

Figure 3. Schematic representation of C9 unfolding polymerization and membrane insertion. (A) Monomeric (globular) C9 reversibly associated with membrane. (B) Unfolding, polymerization, and insertion of three C9 molecules into the membrane—formation of a partial tubule. (C) Formation of a complete tubule and transmembrane channel by continued C9 polymerization (for detail see References 3 and 12).

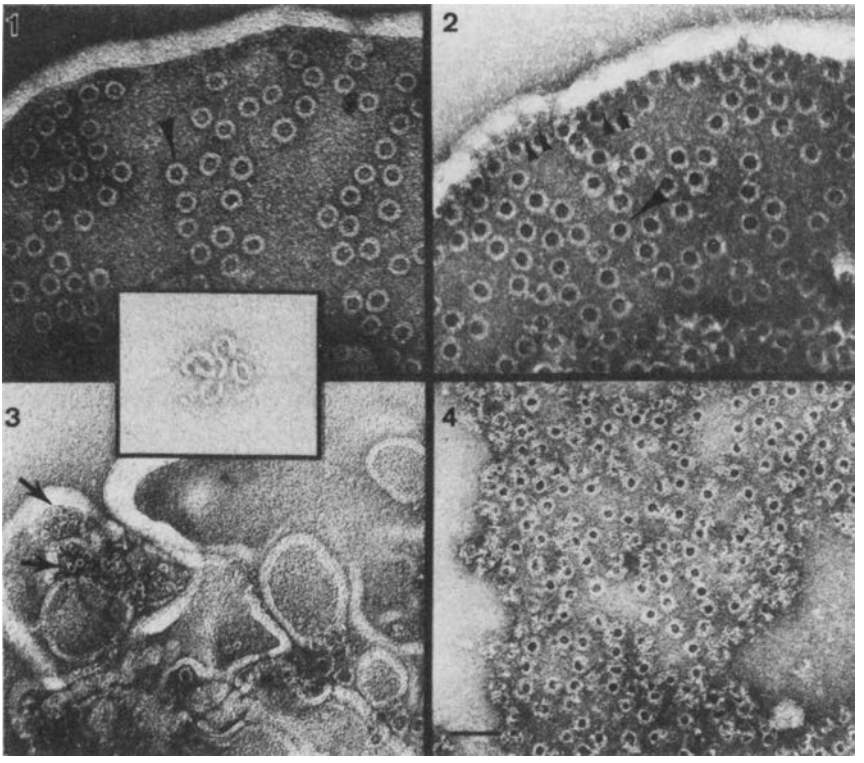

Figure 4. Membrane lesions formed by cytotoxic lymphocytes (panels 1–3) and by complement (panel 4). All panels at the same magnification. (Panels 1 and 2) Murine polymerized perforin 1, internal diameter: ~160Å. (Panel 3) Murine polymerized perforin 2, internal diameter ~70Å. (Panel 4) Human complement lesions, internal diameter ~100Å. (*Inset*) polymerizing perforin 1 (for details see References 2, 4, 5, 8, 9, and 11).

C5 by stoichiometric interaction of C5b (the larger proteolytic fragment of activated C5) with C6, C7, and C8. The C5b-8 complex is inserted into the hydrocarbon core of the membrane. The insertional step takes place when the C5b-6 complex interacts with C7. This seems to involve the unfolding of C7 in a reaction similar to that of the unfolding of C9 during polymerization. The C5b-7 complex as well as the C5b-8 complex are imaged as rodlike structure (Figure 5, panels 10 and 11) of 300–320 Å length. In these complexes C7 forms, at least in part, the membrane binding site, whereas C5b is located on the end of the rod opposite to the membrane. C8 is located adjacent to the membrane in the center of the rod. The C8α-γ subunit appears to be inserted into the hydrocarbon core. C5b-8 mediates slow cytolytic activity which is dramatically enhanced upon binding and polymerization of C9.

The functions of C5b-8 in C9-mediated attack are threefold:

1. The location of C5b-8 determines the site of C9 polymerization and pore formation. The localization of C5b-8 on the target is in turn dependent on the activation function of the classical or alternative pathway of complement.

2. C5b-8 dramatically accelerates C9 polymerization. Spontaneous C9 polymerization at physiologic pH and ionic strength requires 2 days at 37°C; Zn-mediated C9 polymerization requires 2 hours at 37°C whereas C5b-8 induces C9 polymerization within 2 minutes at 37°C.

3. C5b-8 promotes insertion of polymerizing C9 into natural membranes. C9 alone can insert only into artificial protein- and carbohydrate-free phospholipid bilayers. In the presence of C5b-8, however, polymerizing C9 can insert and lyse natural membranes of various organisms including bacteria, virus particles, and tumor cells.

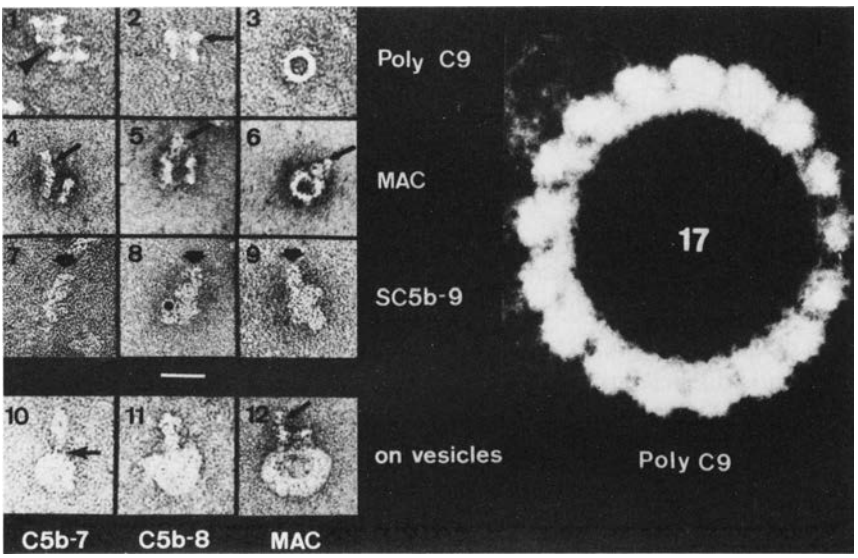

Figure 5. Ultrastructure of various complement complexes. (Panels 1–3) Polymerized C9 in top and side view (*arrow* in panel 1, membrane binding site; in panel 2, poly C9 torus). (Panels 4–6) Membrane attack complex (MAC) in top and side view. (Panel 4) Lateral view. (Panel 5) Frontal view (rotated by 90° along the tubule axis). Note the appendage on the MAC (*arrow*), not present in poly C9, representing the C5b-8 complex. (Panels 7–9) SC5b-9 in three side views; in panel 8 S protein is labeled with colloidal gold. *Arrows* in panels 7–9 point to the C5 subunit in SC5b-9. Note the absence of a tubule structure owing to the inhibition of C9 polymerization by S protein in SC5b-9. (Panel 10) C5b-7 bound to a single bilayer phospholipid vesicle (*arrow* points to membrane binding site). (Panel 11) C5b-8-vesicle. (Panel 12) Membrane attack complex vesicle. (Right panel) Image enhanced poly C9 complex with 17 C9 protomers (for details and further explanations, see Reference 12).

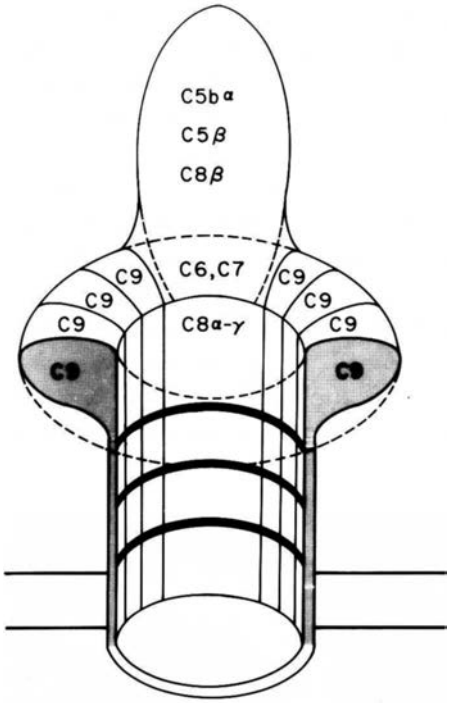

Figure 6. Schematic drawing of the putative arrangements of subunits in the MAC. Note that the tubule is formed by C6, C7, C8α-γ, $C9_{10-16}$. C8 β-chain and C5b are not part of the tubular structure.

The C5b-8 complex during C9 polymerization becomes integrated into the SDS-resistant poly C9 tubule (Figures 5 and 6). Upon boiling in SDS the appendage to the tubule containing the subunits of C5b and the C8 β-chain is dissociated. The tubule structure containing one chain each of C6, C7, C8α-γ, and 10–16 C9 molecules remains intact and has the same structure and dimensions as poly C9.

The mechanisms by which C5b-8 polymerizes C9 is not known. Our current working hypothesis suggests that the C8α subunit, C7, and C6 are homologous to C9 and that they form a C9-like site in the C5b-8. Interaction of the first C9 molecule with this site results in the unfolding of C9, which subsequently interacts with additional C9 molecules in the same manner until the polymerization process is terminated by tubule formation. The proposed homology of C6, C7, C8α-γ, and C9 is supported by antigenic cross-reactions of C6, C7, C8α, and C9 in their reduced form.

Assembly of Polymerized P1 during Cytolytic T Cell Attack

Perforins 1 and 2 are located in the cytoplasmic granules of killer lymphocytes. Isolated granules from several T cell and NK-like cell clones

are highly cytolytically active. Tumor cell lysis by isolated granules is not MHC-restricted and does not display any detectable target specificity. Activation of the cytolytic potential of granules is absolutely dependent on the presence of Ca ions. Granule-mediated lysis is inhibited in the presence of dilute serum (4, 5, 9).

Isolated granules, during the lytic reaction, assemble poly P1 and poly P2 complexes. Preincubation of granules with Ca ions or with Zn results in the polymerization of P1 with a concomitant loss of cytolytic activity; for cytolytic activity to occur, polymerization of P1 has to proceed in the target membrane, because fully polymerized P1 complexes cannot insert into target membranes.

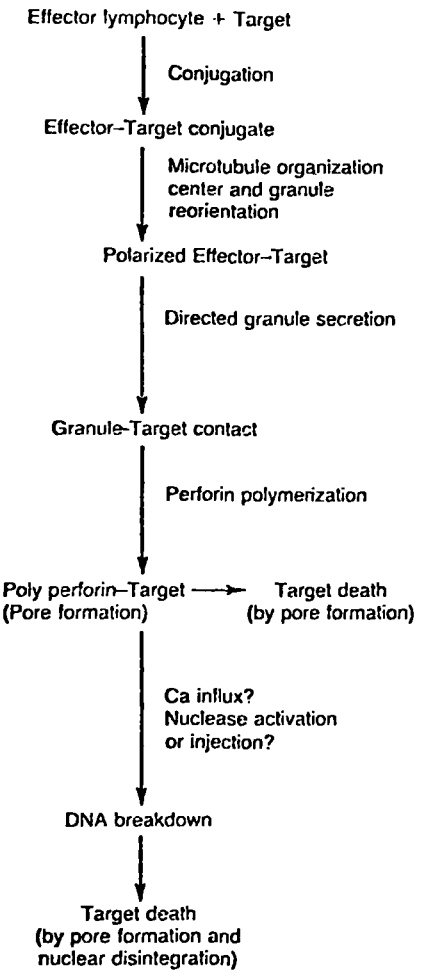

Figure 7. Steps in lymphocyte-mediated cytolysis (for properties of lymphotoxin consult Reference 10).

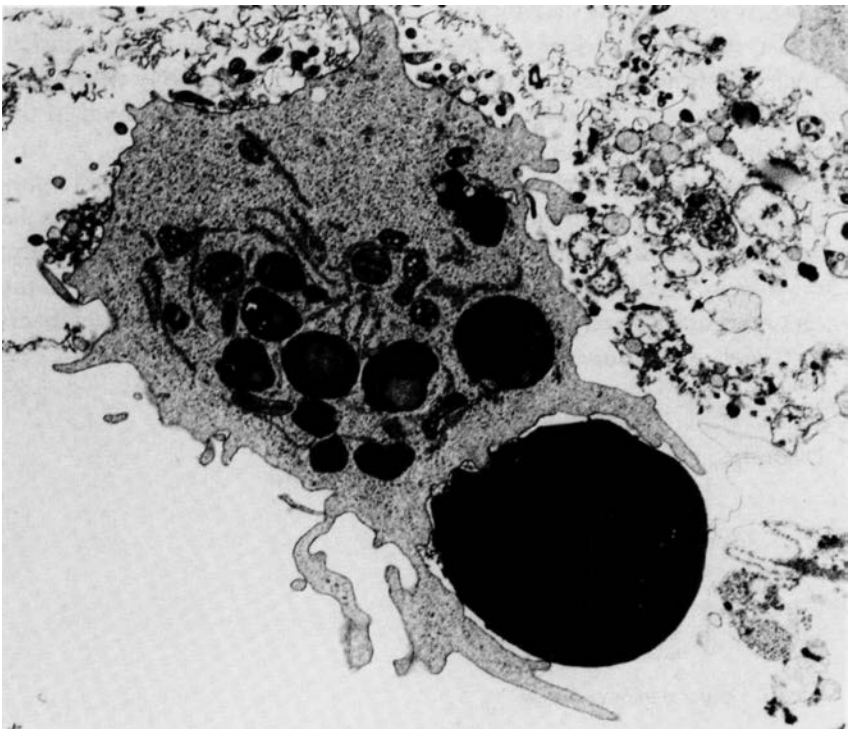

Figure 8. Orientation of granules toward target cell. In this case a cloned NK cell is attacking a rabbit erythrocyte in the presence of antibody (thin-section electron micrograph) (see also References 1 and 4).

The postulated sequence of events for the intact killer lymphocytes to attack a target cell is as follows (Figure 7):

1. *Conjugation.* This step is highly specific and governed by the clonal specificity of the killer lymphocyte. Conjugation is mediated by the antigen receptor and by accessory adherence molecules. Specific conjugation can be bypassed with certain lectins (e.g., concanavalin A). Conjugation can be blocked by antibodies to cell surface receptors.

2. *Killer cell polarization.* The microtubule organizing center is rapidly reoriented in the killer cell to face the contact area. Together with the polarization of the cytoskeletal elements, the granules are brought to the contact site. Polarization of the killer cell is blocked by drugs affecting tubulin polymerization.

3. *Directed granule secretion* (Figure 8). The cytoplasmic granules, containing perforins 1 and 2 and other cytolytic factors (see below), are released in the direction of the target cell into the interstitial space between killer and target and make contact with the target membrane.

4. *Perforin polymerization.* At this point P1 inserts and polymerizes in the target membrane, thus creating large transmembrane pores. It is postulated that the polymerization is triggered by the presence of Ca ions in the interstitial space, however, an alternative activation mechanism for P1 polymerization has not been excluded. Cell death may now ensue due to colloid osmotic lysis and loss of target cell constituents.

5. *Target DNA degradation.* Isolate granules mediated target DNA degradation to multiples of 200 base-pair fragments. Isolated granules thus contain factors triggering DNA degradation, whose identity may be related to lymphotoxin or tumor necrosis factor (see below).

6. *Target death.* Target death occurs as a result of pore formation and nuclear breakdown in the target cells. This lytic mechanism mediated by granules assures destruction of the target cell including its genetic material. This may be vital for combating virus infection or tumor growth.

Use of Membrane Pores for Entry of Toxic Molecules

Polymerized C9 and Lysozyme

In complement-mediated bacteriolysis the effect of the serum lysozyme has been long established (Figure 9). Lysozyme gains access through the poly C9 pores to the peptidoglycan layer underlying the outer membrane and degrades the cytoskeletal elements (Figure 9 C). This results in loss of the structural stability of the bacteria and in the loss of their rodlike morphology. Although the membrane attack complex (MAC) of com-

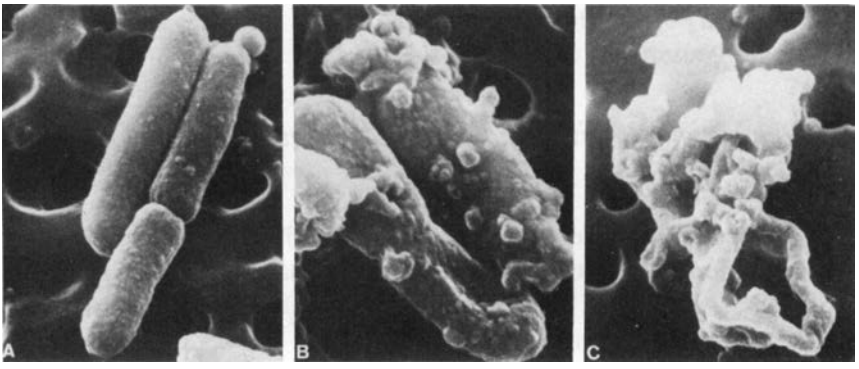

Figure 9. Effect of complement alone and complement plus lysozyme on *Escherichia coli.* (A) Intact untreated *E. coli.* (B) Effect of complement alone; death of *E. coli* accompanied by expansion of outer membrane and blebbing. (C) Breakdown of peptidoglycan and structure of *E. coli* in the presence of complement and lysozyme. (Scanning electron micrographs.)

plement is bactericidal in the absence of other factors, lysozyme, entering through the MAC pores, greatly enhances the cytolytic activity of the MAC.

Polymerized P1 and Lymphotoxin-like Factors

Isolated T cell granules contain an activity that mediates DNA degradation in target cells and kills L cells, the classical target for lymphotoxin and TNF tumor necrosis factor (10). The DNA-degrading activity is detectable in the presence of serum and is not inhibited by preincubation with Ca. These characteristics distinguish the DNA-releasing activity from that of the perforins. In addition, isolated, purified perforin 1 does not cause DNA degradation in target cells killed by its pore-forming activity. These findings demonstrate that granules contain at least two distinct cytolytic activities: the first, the perforin system, attacking the plasma membrane through pore formation, and the second attacking the target nucleus through DNA degradation. The DNA-degrading activity seems to require target cell factors that may be activated by the granule factors (e.g., lymphotoxin).

It is easy to speculate from these observations that the poly P1 system and a lymphotoxin-like activity synergize in granule-mediated target attack. An obvious mechanism for the synergism is the possibility that the poly P1 tubules are utilized by lymphotoxin as entry ports into the target cell. This mechanism would not require the uptake of lymphotoxin via cell surface receptors and endocytotic mechanisms. Thus, even target cells lacking or expressing only in low numbers the putative lymphotoxin receptor could be attacked by lymphotoxin delivered through polymerized perforin pores. Whether this mechanism is correct is subject of current investigation.

Comparison of Perforin 1 and C9 and of Their Polymeric Complexes

Physical Properties

The molecular weight of human C9 is 70,000–73,000 as determined by sedimentation equilibrium analysis. The molecular weight of P1, as judged from SDS-gel analysis, is identical to that of C9 run on the same gel. Unreduced the apparent molecular weight of P1 is lower than that of C9. Both C9 and P1 are rich in disulfides resulting in an increase of the apparent molecular weight upon reduction on SDS-polyacrylamide gels. The disulfide bridges of C9 stabilize the C-terminal and N-terminal domains without linking them together, thus allowing the unfolding of C9 through a putative hinge in the center of the peptide chain. The pri-

mary structure of P1 is not known. Perforin 1 expresses antigenic determinants under reducing conditions also present in reduced C9. A polyclonal antiserum generated to SDS denatured, reduced, and alkylated human C9 detects reduced and alkylated murine P1 in the Western blot analysis.

Polymerization

Both C9 and P1 can be induced to polymerize by low concentration of Zn Cl_2 (100 μM) in the absence of membranes. Polymerization is accompanied by the loss of cytolytic activity because the tubular polymers are inable to insert into membranes. Ca^+ ions effectively polymerize P1 and Ca is believed to be the physiologic effector of polymerization. Ca has some influence also on C9 polymerization. However, C9 is most effectively polymerized by C5b-8 even in the presence of EDTA. Other treatments that polymerize C9 (detergents) have yet not been tested with P1.

Polymerized Perforin 1 and Polymerized C9

The ultrastructures of poly P1 and poly C9 are very similar (Figure 10) (12). The tubules are 160Å long rimmed by a torus on one terminus and by a 40Å-long membrane binding site on the other. Polymerized C9 is composed of 11–20 protomers, and tubules with 14–16 protomers represent 75% of all complexes; the internal diameter of poly C9 varies between 90 and 110Å. The diameter of poly P1 is 160 ± 20Å, suggesting that 20–25 P1 protomers comprise a poly P1 tubule. A similar number of protomers can also be deduced from electron micrographs.

Both tubular poly C9 and poly P1 are resistant to dissociation by boiling in SDS under reducing conditions, and both resist degradation by pro-

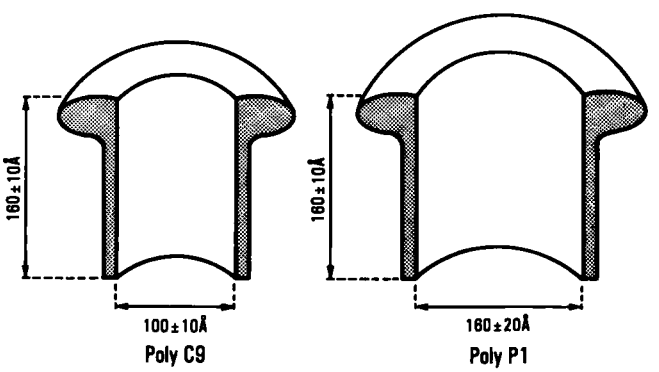

Figure 10. Morphology of poly P1 in comparison to poly C9.

teases. Subunits in the tubules can be covalently linked by spontaneous disulfide exchange.

Functionally, both complexes lyse targets by interfering with the normal permeability barrier function of membranes resulting in colloid osmotic lysis. In addition, the large membrane pores formed by these complexes cause the release of macromolecular compounds from target cell and allow the entry of toxic molecules into target cells. In the latter instance, the tubules serve as delivery system for additional cytotoxic factors that may be even more lethal than the pores alone. In the case of complement, lysozyme functions as accelerator of bacteriolysis, whereas lymphotoxin appears to fulfill an important function in T cell-mediated lysis of virus-infected targets or tumor cells. This combination of pore formation and internal nuclear target degradation insures cytotoxic efficiency.

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Resistance to Infections

T Lymphocyte Function in Recovery from Experimental Viral Infection: The Influenza Model

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Introduction

Infection with most viruses induces a vigorous immune response in their mammalian hosts (1). Because of the importance of type A influenza viruses as major mammalian and avian pathogens, the humoral and cellular immune responses to these viruses have been intensively studied (2). An important issue raised by studies in this and other viral systems is whether cellular immunity contributes to the host defense against, or demise from, viral infection. Early studies of experimental influenza infection demonstrated prolonged survival and reduced pulmonary pathology of influenza-infected congenitally athymic (nude) mice compared to immunocompetent mice. These results implicated T cells in the pathogenesis of influenza disease (3, 4). The failure of such T cell-deficient mice to clear infectious virus, and their eventual death from disseminated viral infecton (5–7), however, suggested a role for T lymphocytes in host recovery. The realization that T lymphocytes were composed of functionally discrete subclasses raised the possibility that virus-specific T cells of different subclasses could mediate either protective or pathologic effects in the infected host.

The discovery by Zinkernagel and Doherty (8) that T lymphocytes from virus-immune mice specifically destroyed virus-infected target cells

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in a major histocompatibility (MHC)-restricted fashion implicated cytolytic T lymphocytes (CTL) as potentially critical effector cells in the control of virus infection (9). Analysis of the CTL response to influenza virus infection strongly supported this contention. In the murine system, anti-influenza cytolytic activity increases early after onset of infection; the rapid rise in pulmonary virus-specific CTL correlates with fall in lung virus levels, which, in turn, is associated with recovery from infection (10). The presence of influenza-specific CTL has also been correlated with viral clearance in humans (11). Direct demonstration that CTL act to limit influenza disease comes from studies showing that adoptive transfer of heterogeneous T cell populations expressing influenza-specific cytolytic activity eliminates pulmonary virus and promotes recovery of lethally infected mice (12, 13). The capacity of transferred CTL to mediate in vivo antiviral activity confirms the role of this T cell subclass in host survival from influenza disease.

A hallmark of the anti-influenza CTL response in both mouse and man is its extensive cross-reactivity for type A influenza virus strains of serologically unrelated subtypes (14–18). Within heterogeneous CTL populations, cross-reactivity has been shown to be mediated by a distinct type A-specific CTL subpopulation (14, 15, 18). Although cross-reactive CTL constitute the major population generated in response to type A influenza infection (19), other CTL populations of more restricted viral specificity are also produced (14, 15). By analyzing influenza recognition by CTL at the clonal level (20), we have uncovered further heterogeneity in the specificity of CTL for type A influenza virus. In addition to isolating cross-reactive CTL clones, CTL clones have been selected that recognize exclusively the immunizing virus strain (unique), virus strains of the same subtype as the immunizing strain (subtype-specific), or shared determinants between virus strains of some but not all type A virus subtypes. The potential therapeutic significance of cross-reactive cellular immunity to type A influenza has stimulated intense interest in evaluating the in vivo antiviral effector function of CTL and defining the viral polypeptides which serve as target antigens for cross-reactive CTL.

To approach these issues, we have studied the activity of influenza-specific cloned CTL populations in experimental influenza infection. The use of CTL clones, rather that heterogeneous CTL populations, has enabled detailed examination of the viral specificity and effector mechanism(s) expressed by antiviral CTL in vivo. Furthermore, the availability of CTL clones with defined in vivo effector function has permitted identification of viral polypeptides that are important in CTL recognition of virus-infected host cells. We have also analyzed the in vitro and

in vivo activities of CTL clones that represent a newly defined anti-influenza CTL subset—class II MHC (H-2I region)-restricted influenza-specific CTL. Comparison of the requirements for presentation of viral antigens to class II-restricted CTL clones and conventional class I-restricted CTL clones has revealed striking differences in viral antigen recognition by these CTL subsets. The implications of these findings for the recognition of virus-infected host cells and the in vivo effector function of antiviral CTL will be discussed.

In Vivo Antiviral Effector Activity of Influenza-specific Cytolytic T Lymphocyte Clones

Previous reports using heterogeneous CTL populations implicated a positive role for CTL in recovery from influenza infection (12, 13). To demonstrate definitively that CTL were, in fact, the effector T cell subset responsible for mediating this protective effect, we analyzed influenza virus-specific CTL cloned (homogeneous) populations for antigen-specific, H-2-restricted antiviral activity in vivo. To carry out this analysis, we selected two cloned CTL lines with different in vitro viral specificities. Clone A4 recognizes and lyses target cells infected with influenza virus strains of the H2N2 subtype (subtype-specific), whereas clone A7 recognizes cells infected with type A influenza viruses of serologically distinct subtypes (cross-reactive). These cloned CTL lines also possess the following properties: (*a*) class I MHC (H-2K/D)-restricted recognition of virus-infected cells; (*b*) antigen- and interleukin 2 (IL-2)-dependent proliferation; and (*c*) Thy 1^+, Lyt 2^+ L3T4$^-$ cell surface phenotype. These characteristics are exhibited by all class I MHC-restricted CTL clones isolated in our laboratory to date (20, 21).

We designed the following adoptive transfer protocol to investigate the in vivo functonal activity of these CTL clones. Normal syngeneic ($H\text{-}2^d$) mice were inoculated with 10 LD_{50} of influenza virus by the intranasal route. At the same time as virus infection, 1×10^7 cells of a CTL clone were injected intravenously into each mouse. As shown in Figure 1, control mice that were infected with 10 LD_{50} of either the A/JAP/57 (H2N2 subtype) virus strain or the A/MEL/35 (H1N1 subtype) virus strain rapidly died from overwhelming viral pneumonia (time to death, 5–8 days). On the one hand, adoptive transfer of either CTL clone A4 or A7 mediated recovery of mice lethally infected with A/JAP/57 virus, the virus strain used to select and maintain these clones in vitro. On the other hand, only A/MEL/35 virus-infected recipients of the cross-reactive CTL clone A7 survived; adoptive transfer of the H2N2 subtype-specific clone

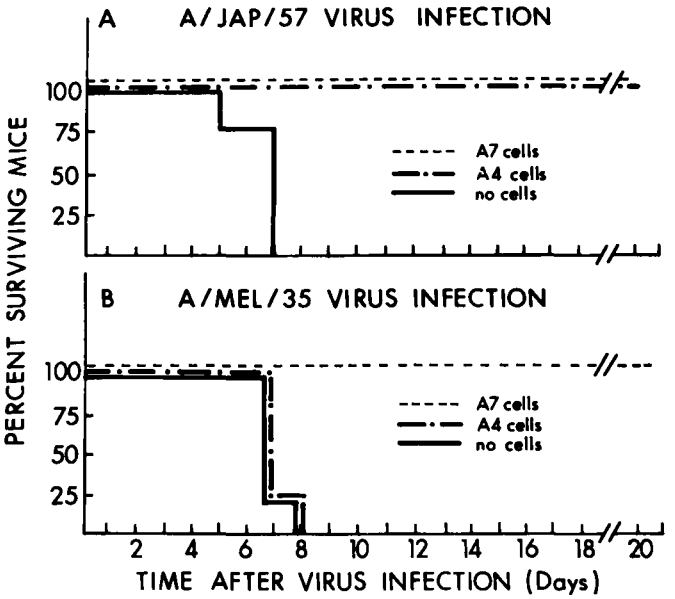

Figure 1. In vivo antiviral activity of CTL clones A4 and A7. Cells (1×10^7) of clone A4 (—·—) or clone A7 (– – – –) were injected intravenously into each BALB/c mouse inoculated intranasally with 10 LD_{50} of either A/JAP/57 virus (A) or A/MEL/35 virus (B). Control infected mice received no cells (———). Each group consisted of four mice. From Reference 46.

A4 failed to alter the outcome of lethal infection from A/MEL/35 virus. The capacity of clone A4 to promote recovery of only the A/JAP/57-infected recipients corresponded to its viral specificity in clearance of infectious pulmonary virus. Clone A4 efficiently reduced the titer of A/JAP/57 virus in the lungs of lethally infected mice (>10^4-fold reduction in infectious pulmonary virus compared to controls) but did not affect the titer of pulmonary A/MEL/35 virus compared to infected control mice. The in vivo protective effect of the clones is also H-2-restricted, in that allogeneic (H-2^k and H-2^b) A/JAP/57-infected recipients of cloned cells died with the same time course as infected control mice. Thus, the viral specificity exhibited by these clones in vitro matches the specificity of their in vivo antiviral activity.

In light of the capacity of these in vitro-propagated CTL clones to promote recovery from lethal infection, it was of considerable interest to examine the in vivo localization of these cells after adoptive transfer. Because respiratory epithelial cells are the principal sites of attack and replication of influenza virus (22), the respiratory mucosa should be an important site of migration of cloned cells in infected recipients. To

examine the in vivo localization of cloned CTL populations, [^{3}H]thymidine-labeled cloned cells were injected intravenously into syngeneic mice lethally infected with influenza virus. Twenty-four hours after cell transfer, mice were sacrificed and the tissue distribution of the cloned cells was assessed by autoradiographic examination of histologic sections. Table I depicts the tissue distribution of cells of CTL clone 14-1 in uninfected mice or mice lethally infected with either A/JAP/57 virus or A/PR/8 virus. Clone 14-1 is an A/JAP/57 virus-specific, class I-restricted CTL clone that mediates recovery of recipient mice lethally infected with A/JAP/57 (H2N2) virus but not A/PR/8 (H1N1) virus. Labeled cells of clone 14-1 were detected in large numbers in the lung, spleen, and mesenteric lymph nodes of uninfected and virus-infected mice, with the majority of labeled cells located in the pulmonary parenchyma. Although clone 14-1 specifically recognizes only A/JAP/57 virus-infected cells, pulmonary infection with either the relevant A/JAP/57 virus or the irrelevant A/PR/8 virus dramatically increased the pulmonary localization of cloned cells. Importantly, significant numbers of labeled cells were also found in the bronchiolar lamina propria, bronchus-associated lymphoid tissue (BALT), respiratory epithelium, and pulmonary lumen. Thus, despite the antigen-nonspecific retention of cells of clone 14-1 in the respiratory tract, these cells migrated into the pulmonary parenchyma and ultimately to epithelial surfaces.

The availability of cloned CTL lines with defined in vivo antiviral activity permitted examination of the effector mechanism(s) utilized by CTL in vivo. Cloned CTL populations have been shown to secrete a variety of soluble factors, most notably interferon-γ, upon MHC-restricted recognition of specific antigen (23–25). We have similarly found that our influenza-specific CTL clones are triggered by appropriate virus-infected histocompatible target cells to release interferon and also to induce local delayed-type hypersensitivity reactions when injected with infectious virus into the footpads of syngeneic mice. These lymphokines could act in an antigen-nonspecific manner to elicit a generalized host defense response. Alternatively, CTL could directly lyse virus-infected cells before infectious progeny virions are released, and thereby limit virus spread. To evaluate whether direct cytolysis or release of soluble mediators represents the predominant effector mechanism expressed by antiviral CTL in vivo, we analyzed the specificity of the in vivo antiviral activity of the H2N2 subtype-specific clone A4. Syngeneic mice were infected simultaneously with A/JAP/57 (H2N2) and A/MEL/35 (H1N1) viruses and then injected with clone A4 or the cross-reactive clone A7. The mortality curves shown in Figure 2 demonstrate that, although adoptive transfer of clone A7 pro-

Table I. Localization of Influenza-specific Cytolytic T Lymphocyte Clone 14-1 in Mouse Tissue by Autoradiography

Virus infection of recipients	*Spleen*	*Mesenteric lymph node*	*Lung*				
			Bronchial lumen	*Bronchial epithelium*	*Lamina propria*	*BALT*	*Parenchyma*
	[³H]thymidine-labeled cells per high power field						
None	135 ± 30[a]	12 ± 3	0	0.2 ± 0.2	1 ± 0.8	1 ± 0.5	144 ± 20
A/JAP/57 (H2N2)	98 ± 16	14 ± 4	0.44 ± 0.3	2.5 ± 0.8	8 ± 1.2	4 ± 0.9	621 ± 67
A/PR/8 (H1N1)	100 ± 25	16 ± 2	0.30 ± 0.3	3.3 ± 1.1	14 ± 2.6	3 ± 0.7	620 ± 45

[³H]TdR-labeled cells (5×10^6) of clone 14-1 were injected intravenously into each syngeneic mouse 48 hours after lethal infection with the indicated type A influenza virus strain. Twenty-four hours after cell transfer, mice were killed and their tissues processed for autoradiography.

a. Values are the means ± SEM from five mice of the frequency of labeled cells in 1×10^3 high power microscopic fields.

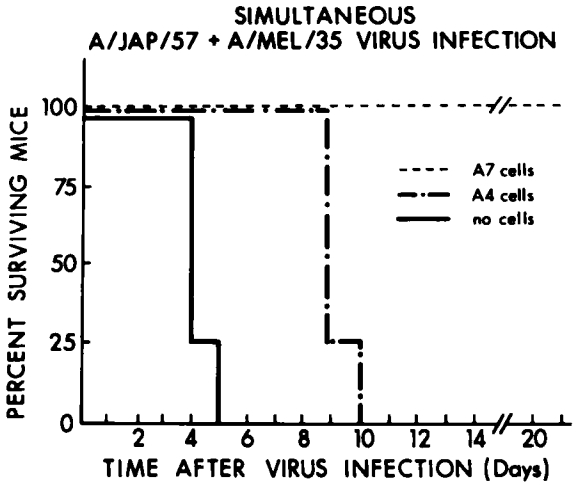

Figure 2. Ability of cloned CTL lines A4 and A7 to promote recovery of mice infected simultaneously with two virus strains of different subtypes. Protocol is as described in Figure 1, except that mice were inoculated simultaneously with 10 LD_{50} A/JAP/57 virus and 10 LD_{50} A/MEL/35 virus. From Reference 46.

motes recovery of dual virus-infected recipients, transfer of clone A4 cannot prevent these mice from dying. Determination of the respective levels of infectious pulmonary A/JAP/57 virus and A/MEL/35 virus showed that clone A4 efficiently cleared pulmonary A/JAP/57 virus but had no effect on the level of A/MEL/35 virus in the dual virus-infected lungs (Figure 3). The ability of clone A4 to selectively inhibit replication of the specifically recognized virus strain (A/JAP/57) without affecting the titer of a bystander virus strain (A/MEL/35) argues in favor of the concept that CTL mediate their antiviral effector activity by direct cytolysis of virus-infected cells.

Identification of Influenza Virus Polypeptides Recognized by Cytolytic T Lymphocytes

Recent advances in the cloning of influenza genes and expression of their protein products in appropriate target cells have made possible precise identification of influenza polypeptides that serve as target antigens for CTL. The influenza hemagglutinin (HA), the major virion glycoprotein expressed by virus-infected cells, has been suggested by a number of studies to represent a target antigen for anti-influenza CTL (26–29). Using L cell ($H\text{-}2^k$) transformants constitutively expressing the A/JAP/57

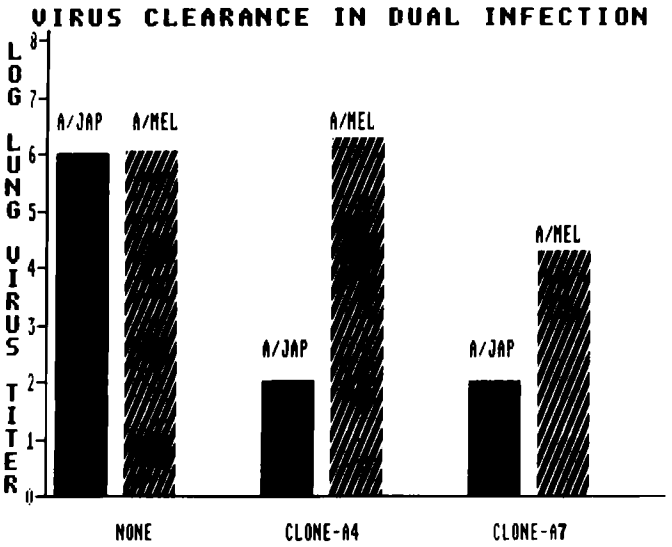

Figure 3. Pulmonary virus titer reduction mediated by CTL clones in mice simultaneously infected with influenza type A virus strains of different subtypes. Adoptive cell transfer protocol is as described in Figure 2. Lung extracts, prepared from recipients 5 days after infection, were treated with anti-A/MEL/35 antiserum (to measure A/JAP/57 virus titer, ■) or anti-A/JAP/57 HA antiserum (to measure A/MEL/35 virus titer, □), and titered by plaque formation on Madin-Darby canine kidney (MDCK) cell monolayers. Virus titers are the log (mean plaque-forming units per milliliter) of lung extracts from three mice. Adapted from reference 46.

virus HA, we directly demonstrated recognition of the HA by both heterogeneous and cloned populations of type A influenza-specific syngeneic CTL (30). The use of L cell transfectant lines, however, limits this kind of analysis to the H-2^k haplotype and to a particular target cell type. To overcome these difficulties, the vaccinia virus has been employed as a eukaryotic expression vector for influenza genes (31). A recombinant vaccinia virus containing the A/JAP/57 HA gene (HA-VV) has been successfully used to vaccinate against respiratory infection with influenza virus (31), to stimulate a HA-specific murine CTL response, and to demonstrate recognition of HA on histocompatible target cells by heterogeneous influenza-specific CTL populations (32). A recent report using another recombinant vaccinia virus containing the nucleoprotein (NP) gene (NP-VV) (33), along with a study using NP-transfected L cell lines (34), have implicated NP as the major target antigen for cross-reactive anti-influenza CTL.

We have employed the HA-VV and NP-VV recombinant viruses to probe our CTL cloned lines with in vivo antiviral activity for recognition of these influenza polypeptides. As shown in Table II, CTL clone 11-1 (A/JAP/57-unique) and CTL clone A4 (H2N2 subtype-specific) specifically lyse target cells infected with HA-VV, and fail to recognize target cells infected with either NP-VV or the parent vaccinia virus (VV). This result confirms the reports cited above that the HA is a target antigen for CTL of restricted viral specificities. In contrast, the cross-reactive CTL clones 14-13 and 19-11 recognize NP-VV-infected target cells, but not HA-VV-infected cells. Thus, both the influenza HA and NP can serve as target antigens for CTL clones that mediate recovery from influenza infection. Importantly, another cross-reactive CTL clone, A7, failed to recognize target cells infected with either HA-VV or NP-VV. This implies that antigenically conserved virion polypeptides other than NP, such as the influenza matrix and nonstructural proteins, may also serve as target antigens for cross-reactive CTL in vivo.

Table II. Recognition of Hemagglutinin and Nucleoprotein Recombinant Vaccinia Virus-infected Cells by Cytolytic T Lymphocyte Clones with In Vivo Antiviral Effector Activity

		Percent specific ^{51}Cr release from P815 ($H\text{-}2^d$) target cells[b]				
CTL clone	*Viral specificity*[a]	*A/JAP/57*	*HA-VV*	*NP-VV*	*VV*	*uninfected*
11-1	A/JAP/57-unique	75[c]	77	0	2	4
A4	Subtype-specific	44	65	0	1	4
14-13	Cross-reactive	39	2	22	1	5
19-11	Cross-reactive	52	2	63	1	4
A7	Cross-reactive	68	2	1	2	9

a. As determined in cytotoxicity and proliferation assays (20, 46).

b. CTL clones were examined for cytolytic activity on ^{51}Cr-labeled P815 target cells that were left uninfected or infected with A/JAP/57 virus, or a recombinant vaccinia virus containing the A/JAP/57 hemagglutinin (HA-VV) or A/PR/8 nucleoprotein (NP-VV) gene, or the parent vaccinia virus (VV). There were 1×10^4 target cells per well. Effector cell/target cell ratio is 5:1. Assay time is 6 h.

c. Values are the means of triplicate wells.

Influenza-specific Cytolytic T Lymphocyte Clones Restricted by Class II Major Histocompatibility Complex Products

A major criterion used to categorize T lymphocytes into functionally distinct subsets is the recognition of antigen in association with different MHC-encoded proteins. In general, CTL are restricted in their recognition of antigen by class I MHC products (encoded by the H-2K, D, or L loci in the mouse and the HLA-A, B, or C loci in the human) (35). Helper T lymphocytes (T_H), in contrast, are restricted by class II MHC (Ia) products (encoded by genes located in the H-2I region in the mouse and the HLA-DR/DC loci in the human) (35). At variance to these traditional MHC restriction patterns for CTL and T_H are several recent studies documenting the existence of alloreactive murine CTL lines (36) and clones (37) directed to class II MHC structures and of H-2I region-restricted antigen-specific CTL clones (38). Antiviral human CTL clones restricted by class II MHC products have also been described in several viral systems (39–41). In particular, we have characterized type A influenza

Table III. Selective Recognition of Ia^+ Target Cells by Antiviral Cytolytic T Lymphocyte Clones

	Percent specific ^{51}Cr release from target cells[b]			
	P815 ($K^+D^+Ia^-$)		*A20-1.11 ($K^+D^+Ia^+$)*	
Clone[a]	*A/JAP/57*	*B/LEE*	*A/JAP/57*	*B/LEE*
A4	54[c]	10	28	3
A7	78	12	35	8
D8	3	2	46	0
G1	6	2	57	6
U5	8	3	25	0
U2	2	1	7	0
U11	3	0	5	0

Adapted from Reference 42.

a. All clones of BALB/c ($H\text{-}2^d$) origin.

b. As in Table II. Effector cell/target cell ratio is 10:1.

c. Values are the means of triplicate wells.

virus-specific human CTL clones whose restriction specificities map to HLA-DR (39).

The existence of class II-restricted anti-influenza CTL in the human prompted us to confirm that class II-restricted CTL were also generated in the mouse in response to type A influenza infection. As shown in Table III, we isolated several influenza-specific T cell clones from A/JAP/57 virus-immune mice that failed to lyse A/JAP/57 virus-infected P815 cells, an Ia^- mastocytoma line widely used as a target cell in cytotoxicity assays. When these clones were examined for virus-specific cytolytic activity against the histocompatible Ia^+ A20-1.11 B cell lymphoma target, three of these five clones (D8, G1, and U5) lysed A/JAP/57 virus-infected A20-1.11 cells with an efficiency similar to that of the conventional class I-restricted CTL clones A4 (K^d-restricted) and A7 (L^d-restricted). None of these clones lysed uninfected or B/LEE-infected A20-1.11 cells. Although clones D8, G1, U5, U2, and U11 expressed the Lyt 1^+2^-, $L3T4^+$ phenotype characteristic of class II-restricted T cells, antigen-specific proliferation by these clones was formally shown to be I-A- or I-E-restricted by conventional genetic mapping with the use of inbred MHC recombinant mouse strains (42). The cytolytic effector function of clones D8, G1, and U5 was also shown to be class II MHC-restricted by selective inhibition with monoclonal antibodies directed to class II MHC products or L3T4 and by lysis of influenza-infected L cells expressing Ia antigens introduced by DNA-mediated gene transfer (42).

The finding that these CTL clones produced antigen-nonspecific factors that helped in vitro B cell respones (42) raised the possibility that their cytolytic effector function was mediated by release of nonspecific cytolytic factors rather than by direct lysis of virus-infected cells. The former possibility is supported by a recent report of nonspecific (bystander) target cell destruction by antigen-stimulated class II-restricted CTL clones (38). This result contrasts sharply with the classical description of class I-restricted CTL-medited cytolysis—inability to kill bystander cells (43). Yet, like class I-restricted CTL, none of the influenza-specific class II-restricted CTL clones, after recognition of target cells expressing appropriate viral antigens, exhibited any cytolytic effect on bystander cells (42). Furthermore, preliminary evidence indicates that both class I- and class II-restricted CTL clones induce rapid digestion of target cell nuclear DNA, a phenomenon characteristic of CTL-mediated lysis (44). This finding, along with the absence of bystander cell killing, strongly suggests that class I- and class II-restricted CTL utilize the same cytolytic effector mechanism—direct lysis of virus-infected cells.

The class II-restricted CTL clones also exhibit considerable heteroge-

neity in viral antigen specificity. Analysis of the fine antigenic specificities of a large panel of influenza-specific class II-restricted CTL clones has revealed the same repertoire of antigenic diversity expressed by class I-restricted CTL clones. Class II-restricted CTL clones were isolated that recognized all type A influenza virus strains (cross-reactive) as well as clones that exhibited more restricted viral specificities (subtype-specific, and unique for the immunizing virus). In addition, the viral specificities exhibited by the class II-restricted CTL clones do not correlate with their restricting MHC elements. Both I-A- and I-E-restricted subtype-specific CTL clones have been isolated, as well as cross-reactive I-E-restricted CTL clones. Infection of target cells with reassortant influenza strains and recombinant vaccinia virus extracts expressing the influenza HA gene product has identified the HA as a target antigen for some, but not all, of the subtype-specific class II-restricted CTL clones (e.g., clone G1). Further mapping of the influenza polypeptides recognized by class II-restricted CTL clones may uncover differences in the viral target antigens to which class I- and class II-restricted CTL are directed.

In Vivo Effector Function of Class II-restricted Cytolytic T Lymphocytes

Leung and Ada (45) had reported enhanced mortality of influenza-infected mice upon adoptive transfer of H-2I region-restricted, influenza-specific, Lyt 1^+2^- T cells. This finding raised the possibility that the class II-restricted anti-influenza CTL clones might mediate a pathologic effect in vivo. To investigate the in vivo activity of the class II-restricted CTL clones, we employed the same adoptive transfer protocol used to assess the in vivo function of the class I-restricted CTL clones (46). Cloned cells (1×10^7) were injected intravenously into syngeneic mice inoculated at the same time with 10 LD_{50} of influenza virus by the intranasal route. The two class II-restricted CTL clones selected for this analysis, D8 and G1, differ in their in vitro viral specificities. Clone D8 exhibits cross-reactive recognition for all type A influenza virus strains, whereas clone G1 is directed to a determinant shared by type A viruses of the H2N2 subtype (42). Figure 4 depicts the mortality curves of recipients of these CTL clones that were lethally infected with either A/JAP/57 (H2N2) virus or A/MEL/35 H1N1) virus. Similar to the class I-restricted CTL clones (Figure 1), adoptive transfer of clone D8 or G1 promoted recovery of mice lethally infected with A/JAP/57 virus, the virus strain used to select these clones. However, only the cross-reactive clone, D8, prevented death of recipient mice from A/MEL/35 virus infection; the mortality curve

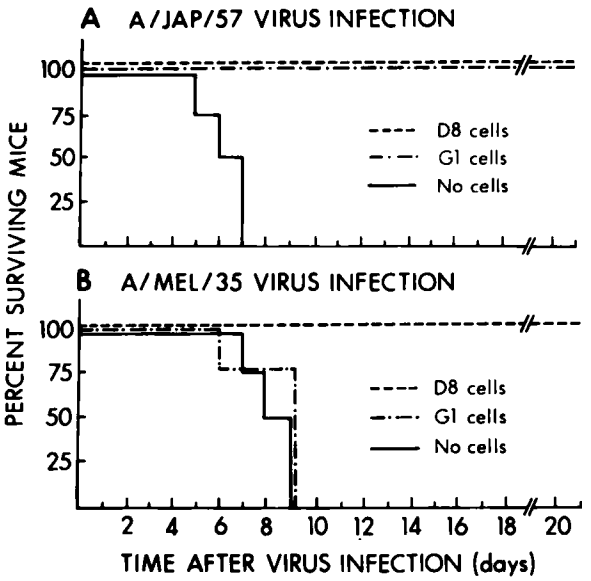

Figure 4. In vivo antiviral activity of class II MHC-restricted CTL clones D8 and G1. Protocol is the same as in Figure 1, except that 1×10^7 cells of clone D8 (————) or 5×10^6 cells of clone G1 (—·—) were injected into lethally infected mice.

of A/MEL/35-infected recipients of the H2N2 subtype-specific clone, G1, was indistinguishable from that of control A/MEL/35-infected mice which received no cells. In agreement with the mortality curves, clone G1 cleared infectious pulmonary A/JAP/57 virus but had no effect on the level of A/MEL/35 virus. Titration of the minimal cell dose required to effect recovery from infection with 10 LD_{50} of A/JAP/57 virus revealed that as few as 3×10^6 cells of either class I- or class II-restricted CTL clones mediated recovery of all recipients. Furthermore, analysis of the time course for clearance of infectious A/JAP/57 virus from the lungs of recipients of either the class I-restricted CTL clone A7 or the class II-restricted CTL clone G1 revealed nearly parallel kinetics of pulmonary virus reduction (Figure 5). Thus, the class II-restricted CTL clones, like the class I-restricted CTL clones, exhibit highly efficient in vivo antiviral activity in an antigen-specific fashion.

The in vivo antiviral effector mechanism (i.e., direct lysis of virus-infected cells vs. lymphokine release) utilized by the class II-restricted CTL was investigated using the dual virus infection model described above. Cells (1×10^7) of the H2N2 subtype-specific, class II-restricted CTL clone G1 were injected into syngeneic mice simultaneously infected with 10 LD_{50} A/JAP/57 virus and 10 LD_{50} A/MEL/35 virus. As reported for

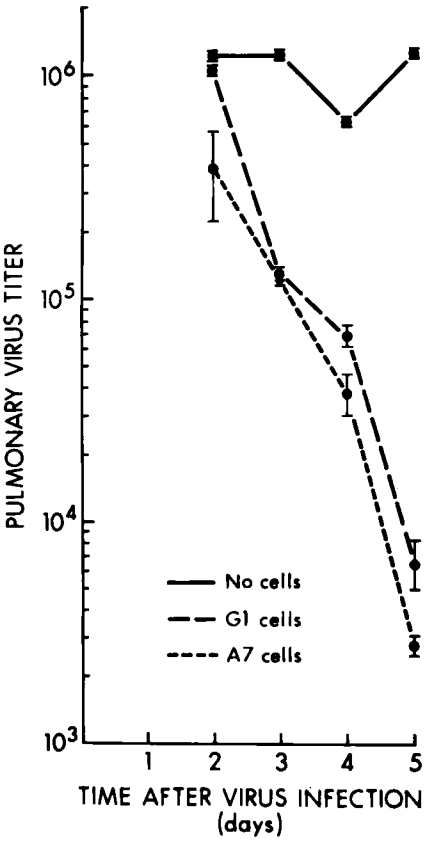

Figure 5. Kinetics of pulmonary virus reduction after adoptive transfer of CTL clones. Cells (1×10^7) of clone G1 (——) or clone A7 (– – –), or medium with no cells (———) were injected into BALB/c mice infected with 10 LD_{50} A/JAP/57 virus. Each point is the mean plaque-forming units ± standard error of the mean (PFU ± SEM) per milliliter of infectious virus in lung extracts from three mice at the indicated times after infection.

the dual virus infection experiment carried out with a class I-restricted, subtype-specific CTL clone (Figure 2), all of the dual virus-infected recipients of clone G1 died, and the mortality curve resembled that of mice infected with A/MEL/35 virus alone. The failure of clone G1 to promote recovery of the dual virus-infected recipients suggests that G1 does not mediate its in vivo antiviral activity by engaging a generalized host defense response. Analysis of the respective titer of infectious A/JAP/57 and A/MEL/35 viruses in the lungs of recipients of clone G1 revealed efficient clearance of A/JAP/57 virus (>10^3-fold reduction in infectious pulmonary virus compared to controls) but no effect on A/MEL/35 levels compared to control dual virus-infected mice. These findings emphasize the exquisite antigen specificity expressed by clone G1 in vivo and argue in favor of the concept that class II-restricted CTL, like class I-restricted CTL, carry out their in vivo antiviral effector function by direct lysis of virus-infected cells.

Viral Antigen Presentation to Class I- and Class II-restricted Cytolytic T Lymphocytes

Over the past decade evidence has accumulated suggesting that antiviral CTL and T_H differ in their requirements for antigen presentation. Early studies showed that sensitization of target cells for lysis by class I-restricted CTL required de novo synthesis of viral proteins (47). This observation has been definitively confirmed in the influenza system in recent studies showing that introduction of the HA gene by infection with a recombinant vaccinia virus (32) or by DNA-mediated gene transfer (30) leads to cell surface expression of the HA and target cell recognition by class I-restricted CTL. These findings suggest that antiviral class I-restricted CTL recognize viral polypeptides in their native state, presumably displayed as integral constituents of the target cell plasma membrane. Helper T lymphocytes (T_H), in contrast, recognize soluble proteins that have been "processed" such that appropriate antigenic determinants are displayed on the surfaces of Ia^+ cells (48). This dichotomy in antigen presentation requirements between CTL and T_H has been well documented in the influenza virus system. In contrast to cells infected with influenza virus, cells pulsed with noninfectious influenza virions are not recognized by class I-restricted CTL (49). Influenza-specific class II-restricted "T helper" cells, however, proliferate in response to spleen cells exposed to noninfectious virus (45). The capacity of T_H to recognize soluble viral proteins is highlighted by recent studies using isolated influenza virion polypeptides and synthetic peptides of the influenza HA to stimulate influenza-specific T_H clones (50–52).

Because the influenza-specific class II-restricted CTL clones share functional properties of both T_H and class I-restricted CTL, we were interested in evaluating their viral antigen presentation requirements. The first indication that the class I- and class II-restricted CTL possess different antigen recognition processes emerged during examination of the capacity of class II-restricted CTL clones to recognize target cells exposed to noninfectious (ultraviolet [UV] light-inactivated) influenza virus. As shown in Table IV, the class I-restricted CTL clone 35-6 efficiently lysed target cells sensitized with infectious A/JAP/57 virus but failed to recognize target cells treated with UV light-inactivated A/JAP/57 virus. In contrast, two class II-restricted CTL clones, G1 and D8, efficiently lysed target cells exposed to either infectious or inactivated A/JAP/57 virus. None of the clones recognized uninfected or B/LEE-infected cells.

The observeration that intact inactivated influenza virus sensitized target

Table IV. Target Cell Sensitization for Class II Major Histocompatibility Complex-restricted Cytolytic T Lymphocyte Recognition by Ultraviolet Light-inactivated Virus

	Percent specific ^{51}Cr release from Ia^+ target cells[b]			
CTL clone[a]	*Infectious A/JAP/57*	*UV-inactivated A/JAP/57*[c]	*Uninfected*	*B/LEE*
35-6 (class I)	70[d]	2	3	4
G-1 (class II)	61	67	7	17
D8 (class II)	48	62	2	6

a. Major histocompatibility complex restriction was mapped using recombinant inbred mouse strains (30, 42) and L cell transfectants expressing class II MHC gene products (42).

b. As in Table II. Effector cell/target cell ratio is 10:1.

c. 2,500 hemagglutinating units of UV light-inactivated A/JAP/57 virus per 1×10^6 target cells.

d. Values are the means of quadruplicate wells.

Table V. Isolated Influenza Hemagglutinin Sensitizes Targets for Class II-restricted Cytolytic T Lymphocyte Recognition

	Percent ^{51}Cr release from Ia^+ target cells[b]		
CTL clone[a]	*Infectious A/JAP/57*	*Isolated HA*[c]	*B/LEE*
11-1 (class I)	40[d]	4	0
G1 (class II)	61	54	3
U-12 (class II)	64	57	3

a. As in Table IV.

b. As in Table II. Effector cell/target cell ratio is 2:1.

c. 300 hemagglutinating units of isolated A/JAP/57 HA per 1×10^6 target cells.

d. As in Table IV.

cells for lysis by class II-restricted CTL raised the possibility that isolated virion polypeptides could also render target cells susceptible to lysis. As shown in Table V, two class II-restricted CTL clones, G1 and U-12,

Table VI. Effect of Chloroquine on Target Cell Sensitization for Cytolytic T Lymphocyte Recognition

CTL clone	*MHC restriction*[a]	*Uninfected*	*A/JAP/57 Infected*	*A/JAP/57-infected + chloroquine*[b]
		Percent specific ^{51}Cr release from A20-1.11 target cells[c]		
14-1	K^d	4[d]	64	66
14-7	K^d	2	66	62
G1	I-E^d	4	68	14
U-12	I-E^d	2	67	7

a. As in Table IV.

b. As in Table II.

c. Target cells were infected in the presence of 5×10^{-5} M chloroquine. Chloroquine was present at 5×10^{-6}M throughout the assay.

d. As in Table IV.

efficiently lysed target cells treated with purified A/JAP/57 virus HA, whereas a class I-restricted CTL clone, 11-1, whose specificity has been mapped to the A/JAP/57 HA using the recombinant vaccinia virus (Table II), failed to recognize HA-treated target cells. This result implies that distinct pathways for presentation of viral antigens dictate whether viral determinants are recognized by class I- or class II-restricted CTL. This conclusion is strongly supported by the ability of chloroquine, a lysosomotrophic agent that blocks antigen-processing events, to abrogate virus sensitization of target cells for lysis by the class II-restricted CTL clones G1 and U-12, but not by the class I-restricted CTL clones 14-1 and 14-7 (Table VI). Complementing this result is the finding that H-2I region-restricted CTL clones G1 and U-12 could not recognize target cells infected with the recombinant vaccinia virus containing the A/JAP/57 HA gene (HA-VV). This eukaryotic expression vector enabled expression of the HA gene in the infected target cell without transfer of the influenza virion-associated HA polypeptide. Clones 14-1 and 14-7, however, lyse HA-VV-infected targets as efficiently as targets infected with A/JAP/57 virus. Taken together, these findings suggest that processing of viral proteins is an essential step in the presentation of viral antigens to class II-restricted CTL, whereas class I-restricted CTL recognize determinants of newly

synthesized viral proteins expressed on the surfaces of target cells as a result of infection.

Conclusions

The findings presented in this chapter establish the existence of two T lymphocyte populations with potent in vivo antiviral activity—class I MHC- and class II MHC-restricted CTL. Cloned populations of each CTL subset efficiently eliminated infectious influenza virus from the lungs of lethally infected syngeneic mice and promoted their recovery. The in vivo effector function of both class I- and class II-restricted CTL exhibit exquisite viral antigen specificity. This finding has important implications with regard to the mechanism through which CTL mediate their antiviral activity in vivo. As reported by us (46) and others (23–25), class I-restricted CTL cloned lines can be triggered by specific antigen to release a variety of nonspecific soluble factors. The influenza-specific class II-restricted CTL clones also release a number of different lymphokine activities (e.g., interferon, IL-2, and B cell helper factors) after antigenic stimulation. While antigen-nonspecific soluble factors may facilitate the antiviral activity of class I- or class II-restricted CTL, the failure of either class I- or class II-restricted CTL clones to control infection by bystander virus supports the concept that both CTL subsets utilize predominantly an antigen-specific antiviral effector mechanism in vivo, most likely direct lysis of virus-infected cells.

The availability of CTL clones with defined in vivo antiviral activity has permitted identification of the influenza virus polypeptides recognized by CTL in vivo. By using target cells that express isolated HA and NP molecules, introduced by recombinant vaccinia viruses containing influenza genes encoding these polypeptides, we have unambiguously mapped HA as a target antigen for CTL clones exhibiting restricted viral specificities (unique and subtype-specific) and NP as a target antigen for type A influenza cross-reactive CTL clones. Although recent studies have implicated NP to be the major influenza polypeptide recognized by cross-reactive CTL (33, 34), we have isolated cross-reactive CTL clones that fail to lyse NP-expressing target cells and yet efficiently mediate recovery from lethal influenza infection. Thus, viral polypeptides that, like NP, are antigenically conserved across type A influenza subtypes, such as the influenza matrix and nonstructural proteins, could serve as target antigens for cross-reactive CTL. Similarly, a number of subtype-specific CTL clones that exhibit in vivo antiviral activity are also directed to viral antigens other than HA or NP. Taken together, these results suggest that viral

polypeptides in addition to HA and NP are expressed on the surfaces of infected host cells and are recognized by CTL.

Evaluation of viral antigen presentation to class I- and class II-restricted CTL has revealed radical differences in the requirements for antigen recognition by these CTL subsets. Sensitization of target cells for recognition by class I-restricted CTL clones required expression of newly synthesized viral polypeptides on the surfaces of infected cells. In marked contrast, class II-restricted CTL clones recognized target cells treated with noninfectious influenza virions or isolated viral polypeptides, but not target cells expressing newly synthesized viral proteins as a result of infection. The discovery that virus sensitization of target cells for recognition by class II-restricted CTL clones, but not class I-restricted CTL clones, is blocked by the lysosomotrophic agent chloroquine suggests that class II-restricted CTL recognize only processed influenza proteins. These findings further imply that class I- and class II-restricted CTL are directed to mutually exclusive viral antigenic determinants.

The capacity of class II-restricted CTL clones to mediate antiviral activity in vivo raises interesting questions concerning the nature of the target cell recognized by these CTL. First, expression of in vivo effector function by class II-restricted CTL necessitates the existence of virus-infected cells that bear Ia antigens. However, the primary site for influenza virus replication is the respiratory epithelial cell (53), an Ia^- cell type. The most likely explanation for this paradox is induction of Ia antigens on virus-infected respiratory epithelial cells. This possibility is strengthened by evidence that interferon, which induces Ia antigen expression on various cell types in vitro (54), is detected in the lungs of influenza-infected mice within 1-2 days after infection (55) and is produced by antigen-stimulated class I- and class II-restricted CTL. A second, perhaps more complex issue, involves the observation that class II-restricted CTL recognize only processed viral antigens. Thus, in order for these CTL to clear infectious pulmonary virus, appropriately processed viral determinants must be expressed by virus-infected cells. Perhaps respiratory epithelial cells take up plasma membrane fragments of neighboring virus-infected cells, a possible source of noninfectious viral polypeptides. Another possibility is that separate intracellular pathways exist for the expression of viral antigens by infected cells, such that both processed and native viral polypeptides are expressed on the infected cell surface.

In summary, we have demonstrated that two CTL subpopulations, class I MHC-restricted CTL and class II MHC-restricted CTL, express efficient in vivo antiviral effector activity which is highly antigen-specific. Although the relative contribution of each CTL subset to host defense

against viral infection is presently unknown, evidence presented here suggests that induction of either class I- or class II-restricted CTL would play a positive role in recovery from viral disease. In this connection, the finding that class I- and class II-restricted CTL recognize different forms of viral antigen has important implications for influenza vaccine design.

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Selective Immunologic Defect Allowing Persistent Virus Infection and Its Correction with Specific Immunotherapy

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The study of any one virus is designed to provide specific information as to how that virus is put together structurally, replicates, and causes injury within a permissive cell or host. Occasionally, observations uncovered while studying a particular virus may also provide fundamental principles not only for the general area of virology but also to parallel disciplines. This has occurred with the study of lymphocytic choriomeningitis virus (LCMV). Indeed, work with LCMV has provided novel concepts pertaining to virology as well as to immunology and pathology. In this chapter I will attempt to bring together the major tenets resulting from investigations of LCMV, and supply current information that defines the molecular basis of how virus persists and the role the immune system is expected to play in clearance of viral materials.

Lymphocytic choriomeningitis virus first attracted attention after Eric Traub (1) described persistent LCMV infection in adult mice. Traub noted that such persistently infected adult mice appeared clinically healthy yet carried high titers of virus in their blood and organs. However, the LCMV carried by such persistently infected mice when transferred intracerebrally to uninfected adult mice led promptly to an acute infection culminating in death. Later Hotchin (2) noted that, whereas inoculation of virus into uninfected adults led to death, a similar intracerebral dose given newborn mice caused persistent infection with a clinical picture similar to that in the naturally occurring persistent infection of mice described by Traub.

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These data led to two general hypotheses formulated to account for virus persistence per se and the related inability to clear virus from tissues. The first focused on dysfunction of the immune system, a system that normally clears infectious materials in a specific manner from a host. The second hypothesis addressed the possible generation of unique viral variants that are responsible for the differences observed. Burnett crystallized the immune dysfunction hypothesis in his theory on immunologic tolerance. Further support followed when investigators failed to find antibody to LCMV in persistently infected mice and when Volkert and Hannover-Larsen (3) noted that, after the transfer of immune spleen cells into persistently infected mice, antibody to LCMV was detectable and the virus disappeared from many organs. However, the concept of tolerance as the cause was challenged by Oldstone and Dixon (4), who showed that persistently infected mice were not truly immunologically tolerant but indeed made specific immune responses to the virus. Their work clearly showed that the excess of virus and viral antigen present removed free antibody from the circulation to form immune complexes containing the antiviral antibody bound to the virus. This work had important biologic implications, for it indicated that tissue injury occurring in mice persis-

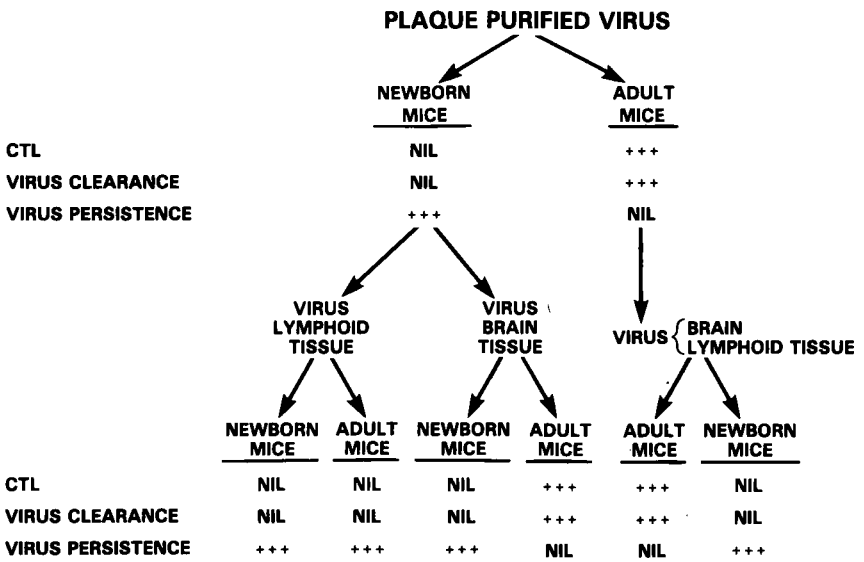


Figure 1. Lymphoid cells from the spleen selectively generate LCMV variants that inhibit the generation of LCMV-specific, H-2-restricted CTLs and help lead to the establishment and maintenance of persistent infection. Schematic representation of data presented in Ahmed et al. (11).

Table I. Lymphocytic Choriomeningitis Virus-specific H-2-restricted Cytotoxic T Lymphocyte Killing Is Selectively Diminished during Persistent Lymphocytic Choriomeningitis Virus Infection

		Percent specific ^{51}Cr release						
		Virus infected H-2^d				*LCMV infected*		
		LCMV		*PICHIND*	*UNINF*	*H-2^d*		*H-2^b*
Status of H-2^d BALB/W mouse	*No.*	*50:1*	*5:1*	*50:1*	*50:1*	*50:1*	*5:1*	*50:1*
Mock infected	1	8	2	8	5	8	2	4
	2	3	0	9	7	3	0	2
	3	5	3	2	6	5	3	3
	4	6	2	5	1	6	2	5
Acute LCMV	1	81	28	7	6	81	28	6
infection	2	76	26	8	4	76	26	7
(day 7 P° SPL)	3	76	12	5	3	76	12	4
	4	61	18	8	0	61	18	1
Persistent LCMV	1	6	3	4	5	6	3	2
infection	2	16	5	9	0	16	5	4
	3	8	4	3	1	8	4	6
	4	12	5	4	2	12	5	0
Persistent LCMV	1	8	3	54	4	8	3	4
infection	2	7	2	63	3	7	2	2
PICHIND day	3	14	5	58	5	14	5	1
7 P° SPL)	4	12	4	46	1	12	4	1

Results from standard ^{51}Cr release assay. Four mice were used in each group, effector-to-target cell ratio was 50:1 or 5:1, assay was for 4–6 hours, and samples were run in triplicate with variance of less than 10%. Target cells used: H-2^d, BALB/clone 7; H-2^b, MC57.

tently infected with a virus like LCMV was likely caused by the specific immune response against the virus rather than other postulated mechanisms such as a response versus self components (2). An additional implication of the Oldstone and Dixon finding was that other kinds of poorly understood persistent infections were probably associated with similar

Table II. Data Revealed by Study of Lymphocytic Choriomeningitis Virus-specific H-2-restricted Cytotoxic T Lymphocyte Clones

In vitro cloned CTLs

1. Can control virus spread and reduce viral titer in the absence of any other accessory cell.
2. Are highly efficient; a ratio of 2.5 CTLs to 1 target cell is capable of lysing more than 90% of target cells within 4–6 hours.
3. Recognize N and C_1 domains of H-2.
4. Recognize mainly conformation changes in H-2.
5. Have an H-2^{dd}-restricting element that can see variable domains among LCMV strains.
6. Have an H-2^{bb}-restricting element that sees mainly common domains among LCMV strains.
7. Generate interferon-gamma that is not directly associated with lytic activity.
8. Kill without generation of interleukin 2.
9. Produce lesions of internal and external diameters of 145 ± 25, 230 ± 25 Å, respectively, in membranes of H-2-restricted, virus-specific target cells.
10. Are separable from splenic CTLs on the basis of monensin sensitivity; monensin blocks lysis by splenic CTLs but not by cloned CTLs.
11. Are induced and recognize viral proteins encodes by the small RNA chromosome.

events, that is, the relative absence of free circulating antibody, the presence of circulating immune complexes, and the localization and trapping in tissues of the specific infectious virus–host antibody immune complexes. Indeed, glomerulonephritis and arteritis were soon identified as frequent pathologic manifestations of persistent infections resulting from the trapping of virus–antibody immune complexes previously formed in the circulation (reviewed in References 5 and 6). Of broader biologic significance, these observations with LCMV provided the model for establishing that immune complexes formed during several microbial infections, including those with other RNA and DNA viruses, bacteria, and parasites, and that persistent infection did not lead to an "immunologic tolerant state." One example is the situation in which the appropriate

Table II continued

In vivo cloned CTLs
1. Reduce virus titer in spleen by 3–5 logs in acute infection within 8 hours of transfer.
2. Maintain reduced virus titers (less than 50 plaque-forming units detected which is limit of assay) in spleens for up to 5 days observation.
3. Need H-2 restriction to work and are virus specific.
4. Approximately 3×10^6 or more CTLs are required.
5. Show unique migratory pattern when compared to nonimmune spleen cells or CTL clones that are neither LCMV specific nor appropriately H-2 restricted.
6. Cause specific acute immunopathologic injury leading to death when transferred to syngeneic mice persistently infected with LCMV. Such events are caused by approximately 3×10^6 or more CTL.

Data were compiled from the following references: Byrne et al. (20), Byrne and Oldstone (23), Ahmed et al. (24), Anderson et al. (25), and Riviere et al. (26).

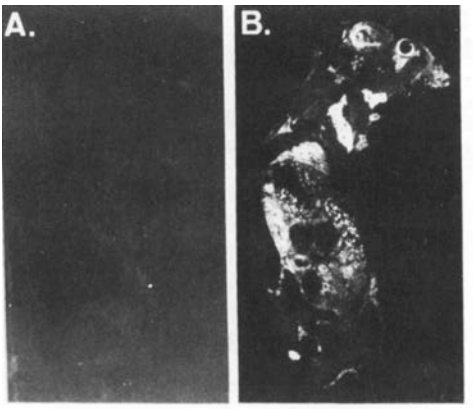
retrovirus-antibody to retrovirus-immune complexes has been found in spontaneously occurring and laboratory-induced retrovirus infection of mice (7, 8). Such results have been confirmed in other laboratories (9) and in recent work with retrovirus infections of humans (10).

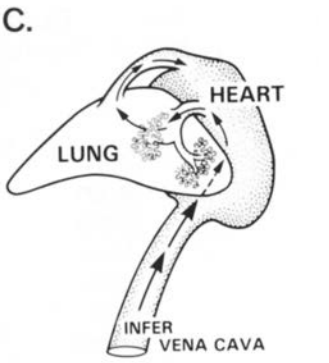
Recently, Rafi Ahmed and his colleagues (11) found that LCMV variants are selectively generated in lymphocytes of splenic tissue in adult persistently infected mice that have been infected since birth. These variants, when inoculated into healthy adult mice, cause persistent infection (Figure 1). The difference in biochemical structure between the original LCMV wild-type inoculum and variants made in the spleen and presumably other lymphoid tissue is currently unknown but under active investigation. These lymphotrophic variants fail to induce the generation of LCMV-specific, cytotoxic T lymphocyte (CTL) responses, and this malfunction is likely one of the major factors in the maintenance of viral persistence. Do other viruses behave similarly, i.e., generate variants that block immune responses in association with persistence? Current investigation is under way with persistent virus infections in man

like those caused by hepatitis B virus and cytomegalovirus to answer this question.

About 15 years ago, we (13) postulated that the fundamental difference between tissue injury after acute and persistent LCMV infections was not a quality of the infective mechanisms but rather of the time frame and vigor of interaction in the virus–host immune response. This hypothesis came from two observations. The first was our finding that persistently infected hosts could and did mount specific antiviral immune responses. These specific immune responses, especially those resulting in formation of immune complexes, were the primary cause of the tissue injury in such persistently infected animals. However, mice persistently infected with LCMV make poor LCMV-specific, H-2-restricted CTL responses. Yet such persistently infected mice can generate virus-specific, H-2-restricted CTLs when challenged with a virus different from LCMV indicating that the defect is a selective and specific one. Table I records these events and compares the level of CTL responses generated in either an acute or a persistent virus infection. The second observation was the classic work of Wally Rowe (13) establishing that tissue injury during acute LCMV infection resulted directly from the host immune response to the virus. Indeed, before we knew that lymphocytes were divided into two major classes, B and T, and that T lymphocytes had specific helper functions for both B lymphocyte differentiation (antibody production) and generation of effector CTLs, Rowe showed that obliteration of immune responsiveness by x-ray treatment, drugs, or thymectomy prevented the acute disease caused by LCMV infection. Subsequent studies by others using this model provided the first evidence for virus specific CTL in vitro (15, 16) or in vivo (17, 18). Precise definition of the requirement for virus specificity and H-2 restriction (19) in CTL activity also came from studies with LCMV. This "H-2 restriction phenomenon" has had broad implications for other cell–cell recognition phenomena well outside of the jurisdiction of virology and is now a major principle of contemporary immunology and cell biology indicating again the wealth of scientific information that has come from studies with LCMV.

Because studies with T cells, including phenotypically marked CTLs in mice and humans, involve the use of heterogeneous populations of lymphocytes, it remained for cloned CTLs to determine the precise activity of such LCMV-specific immune cells. Work during the last two years with cloned CTLs (20–23) has documented the activity of LCMV-specific, H-2-restricted CTL clones in vitro and in vivo. The results of several of these studies are listed in Table II. Inasmuch as a primary defect in persistent infection is the absence of effective and efficient LCMV-specific,





D.

CTL CLONE	H-2 RECOG	MICE PERSISTENTLY INFECTED: LCMV $K^bI^bD^b$	LCMV $K^dI^dD^d$	PICHIND $K^bI^bD^b$
11-2	$K^bI^bD^b$	11/11	0/3	0/4
11-5	$K^bI^bD^b$	3/3	0/3	ND
11-13	$K^bI^bD^b$	3/3	0/3	0/3

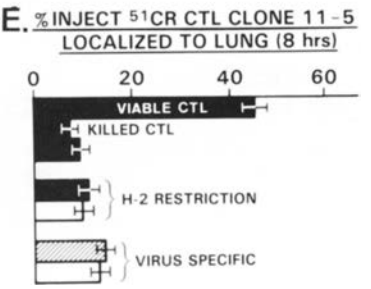


Figure 2. Adoptive transfer more than 3 × 10^6 LCMV-specific, H-2-restricted cloned CTLs into persistently infected mice leads to acute immunopathologic disease and death. (A and B) The distribution of LCMV antigens in an uninfected (A) and persistently infected (B) mouse. A 30-μm whole body animal section was transferred to a nylon membrane, reacted with guinea pig antibody to LCMV, rabbit antibody to guinea pig Ig, and ^{125}I-Staph protein A. A photomicrograph of the resultant autoradiograph is shown. Cytotoxic T lymphocyte clones were administered intravenously via the tail vein and panel C cartoons the return venous flow. Acute immunopathologic injury was recorded as early as 4–6 hours after adoptive CTL transfer with histologic and immunochemical manifestation being primary in the lung. (D) The experimental data recording the ratio of the numbers of animals dying of immunopathologic disease over the total number of animals inoculated. Migration and or accumulation of cloned CTL is LCMV antigen specific and H-2 restricted as shown in Panel E. Upper set of bar graphs shows that adoptive transfer of 1 × 10^6 viable cloned LCMV-specific, H-2 (H-2^b)-restricted CTLs labeled with ^{51}Cr (■) have unique localization patterns in LCMV persistently infected mice differing from the trafficking pattern of these CTLs in (H-2^b) uninfected controls. Middle set of bar graphs shows that CTL clones that were not H-2 (H-2^b CTL → H-2^d mice) restricted but LCMV specific (■) have a trafficking pattern uniquely different from that observed with LCMV-specific, H-2-restricted CTLs in syngeneic LCMV persistently infected mice, but similar to uninfected H-2-restricted mice (□). The lower set of bar graphs shows virus specificity. H-2^b CTL clones not sensitized to LCMV but to H-2^d circulate (▨) in H-2^b persistently infected mice in the same way that they traffic in uninfected H-2^b mice.

Clearance of LCMV Infectious Virus
After I.V. Transfer of LCMV Specific Immune Lymphocytes

Tissue	LCMV titer (log_{10} PFU/organ or ml) PI Untreated	120 days Post Transfer
Serum	4.5	<1.6
Spleen	5.8	<1.6
Liver	5.3	<1.6
Lung	5.3	<1.6
Brain	5.2	1.9
Kidney	6.5	3.9

Clearance of LCMV Nucleic Acid Sequences
After I.V. Transfer of LCMV Specific Immune Lymphocytes

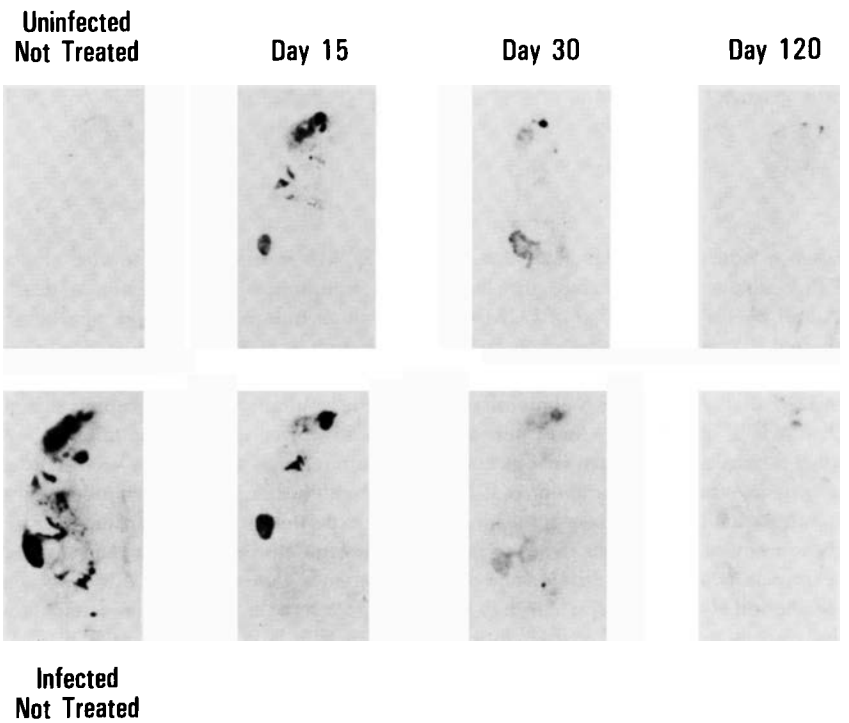


Figure 3. Transfer of immune spleen cells to syngeneic persistently infected mice results in the clearance of infectious virus and viral genetic material. Amount of infectious virus was determined by a plaque assay on Vero cells. Presence of viral genetic material was determined by in situ hybridization to whole mouse sections using a ^{32}P-labeled complementary DNA probe specific for the S chromosome of LCMV Armstrong. From Ahmed et al. (27). Reproduced by permission of the publisher.

H-2-restricted CTLs, initial attempts were made to reconstitute persistently infected mice with these cells. Figures 2 and 3 show the topography of LCMV antigen expression in such infected mice. Figure 2 also records the specific migratory and accumulatory patterns of ^{51}Cr CTL clones and the fact that these clones caused acute immunopathologic injury and death on inoculation. However, the transfer of immune memory lymphocytes (harvested 30–60 days from the spleen after an initial primary immunization) efficiently and effectively cleared infectious virus and viral nucleic acid materials from persistently infected mice (Figure 3) (27, Oldstone, et al. manuscript submitted for publication). Other experimental data indicates that the effector lymphocytes are Th-1.2$^+$, Lyt-2.2$^+$, and Lt34$^+$ and are restricted primarily to class I major histocompatibility complex antigens H-2^d, H-2^l. Thus, the defect in CTL-mediated clearance of virus materials owing to LCMV-induced lymphotrophic event(s) leads, in part, to causing and maintaining persistence but can be corrected by means of selective and specific immunotherapy. We would predict that such immunotherapy will be used in the future to correct selective defects sure to be found as a result of other viruses interacting with lymphocytes. When this occurs, LCMV will have again made a major contribution toward our understanding of important biologic events.

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Role of T Cell Subpopulations and Interleukin 2 Receptors in Cell-mediated Immunity to Facultative Intracellular Bacteria

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Introduction

Infections by facultative intracellular bacteria (table I) are characterized by the formation of granulomatous lesions in infected tissues, macrophage activation, and delayed-type hypersensitivity (DTH). The function of the granulomas appears to be mainly that of trapping the pathogens. In these lesions, macrophages become activated and, in this way, acquire a heightened antibacterial activity that enables them to deal more efficiently with the bacterial parasite; for review, see Hahn and Kaufmann (1). For a long time, little was known about the underlying mechanisms of granuloma formation and macrophage activation. Likewise, the role of DTH in immunity to these infections remained a matter of dispute (2). What was unclear was whether DTH plays a causative role in cell-mediated immunity (CMI) to facultative intracellular bacteria or whether DTH is a phenomenon that occurs at about the same time that protection becomes measurable, but bears no causal relationship to the latter.

In 1969 Mackaness (3), using the murine listeriosis model, showed that macrophage activation and granuloma formation result from specific immune reactions between viable specific lymphocytes and antigen. His conclusions were based on the results of adoptive transfer experiments in which viable lymphocytes from immune animals were able to transfer specific CMI to nonimmune recipients. Antibodies, on the other hand, had no such effect. By 1973, it was established by North (4) and other investigators (5, 6) that the lymphocytes transferring such protection are T cells. Zinkernagel and Doherty (7) showed that in mice recognition of antigen by T cells is restricted by the major histocompatibility gene locus (MHC). With respect to CMI to facultative intracellular bacteria, Zinkernagel et al. (8) claimed that T cell–antigen interactions in CMI

Table I. Facultative Intracellular Bacteria

Mycobacterium tuberculosis
Mycobacterium Leprae
Brucella species
Listeria monocytogenes
Erysipelothrix rhusiopathiae
Yersinia species
Francisella
Salmonella typhi
Salmonella paratyphi
Treponema pallidum
Legionella pneumophila

to facultative intracellular bacteria in mice are restricted by products coded by the H-2 I locus. On the other hand, Cheers and Sandrin (9) provided evidence that protection in whole animals is restricted by the H-2 K locus. As to the interaction between specific T cells and cells of the mononuclear phagocyte system, lymphokines produced by antigen-activated T cells were shown by several authors (10–13) to act as mediators that activate macrophages and entice monocytes to leave the circulation and to accumulate in the periphery and thereby form granulomatous lesions.

The intent of this chapter is to summarize data on the role of T cell subpopulations in granuloma formation and macrophage activation, and to describe, using the listeriosis system as a model, the role of the interleukin 2 (IL-2) receptor in the regulation of T cell–antigen interactions in vitro. Our findings and analysis show that the phenomena occurring during infection with *Listeria monocytogenes* are differently regulated and are induced by different T cell subpopulations.

T Cells and Delayed Hypersensitivity and Specific Immunity

Both DTH and specific immunity to the facultative intracellular bacterium, *L. monocytogenes*, can be transferred by specific T cells from peritoneal exudates induced in mice with a 7-day-old *Listeria* infection. In 1979, it was shown (14) that T cells that transfer protection and DTH are sensitive to in vitro treatment with an antiserum, either Ly-23 or Ly-1, plus complement. Moreover, recombination of the two negatively selected T

Table II. Ly Phenotype of T Cell Subsets Involved in Antibacterial Protection and Delayed-type Hypersensitivity[a]

Group	*Treatment of PETLEs*	*T cell subset*	*Log_{10} protection*	*DTH (0.1-mm footpad swelling)*
1	Nil	Unselected	3.68	8.8
2	C	Unselected	2.59	9.2
3	Anti-Thy-1.2 + C	No	0.65	2.4
4	Anti-Ly-1.2 + C	Ly 1^-23^+	0.62	2.2
5	Anti-Ly-2.2, 3.2 + C	Ly 1^+23^-	0.78	4.4
6	Mixture (1:1) of groups 4 and 5	Ly 1^+23^+	0.64	4.0
	+	Ly 1^+23^-		
7	No PETLEs	—	—	2.2

Abbreviations: C, complement; PETLEs, peritoneal exudate T lymphocyte-enriched cells.

a. PETLEs were treated with anti-Ly antisera plus C, and equivalents of 3×10^6 PETLEs were injected i. v. into recipients which received live *L. monocytogenes* i. v. for evaluation of protection, or received soluble listerial antigen s. c. for evaluation of DTH. Data taken from Kaufmann et al. (10).

cell populations did not result in restoration of the original protective effect (Table II). Because it was then believed that the Ly-1 T cell marker is exclusively expressed on class II antigen-restricted helper T cells, and that Ly-23 is expressed on class I antigen-restricted T cells of the supressor and cytotoxic types, it was therefore concluded from these experiments that T cells transferring DTH and protection carried both the Ly-1 and Ly-23 markers, i.e., that these cells were of the Ly-123 phenotype (14) (Table II). It could not be decided, however, from these experiments whether Ly-123 T cells alone suffice for the induction of protection and DTH, or whether T cells of a different phenotype are required in addition.

Later, Ledbetter et al. (15) produced evidence that Ly-1 is not exclusively expressed on T helper cells, but on Ly-23 cells as well, albeit to a lesser extent. Furthermore, a newly detected T cell marker, L3T4 (16, 17), appears to be exclusively expressed on class II antigen-restricted T cells of the helper type. Therefore, antiserum against the L3T4 marker is a better-suited tool for differentiating class I and class II antigen-restricted T cells. Using antiserum against L3T4 plus complement, the question of which subpopulations are crucially involved in DTH and protection in CMI to *L. monocytogenes* was again tackled, this time using antiserum to L3T4 as well as anti-Ly-2 antiserum plus complement (18; Sperling et al., unpublished results). *Listeria*-specific, peritoneal exudate T lymphocyte-enriched cells (PETLEs) negatively selected by anti-L3T4 antiserum plus complement neither transferred protection nor DTH. On the other hand, and in contradistinction to our earlier experiment employing antiserum to Ly-1, reconstitution of the capability to transfer both protection and DTH was effected by admixing specific T cells that had been treated with anti-L3T4 and with anti-Ly-2 antisera (Table III). Thus, from this experiment, is became clear that two T cell populations, one of L3T4, and the other of the Ly-23 phenotype, are needed for the establishment of effective CMI to *L. monocytogenes*.

T Cell Subpopulations in Granuloma Formation

Further support for the hypothesis that at least two T cell subpopulations are involved in CMI to *L. monocytogenes* came from experiments by Näher et al (19). These authors were beleaguered by the discrepancy between Zinkernagel's observation that CMI is restricted by the H-2 I locus (8) and Cheer's finding that H-2 K restriction is crucial in CMI (9). Why does one not assume that there are differently regulated T cell populations involved? Encouragement for asking this question came from work by Woan and McGregor (20), who had shown that a crucial T cell population for protection resides in the cytotoxic T cell pool.

Table III. Effect of Anti-Ly-2 and Anti-L3T4 Antisera Plus Complement Treatment on the Capacity of L. monocytogenes-immune Peritoneal Exudate T Lymphocyte-enriched Cells to Transfer Systemic Antibacterial Protection

Group	*Treatment of specific PETLEs*[a]	*Log_{10} viable bacteria in spleens*[b]	*Log_{10} protection in spleens*[b]
1	Complement	6.4 ± 0.4	1.5
2	Anti-L3T4 and complement	7.4 ± 0.4	0.5
3	Anti-Ly-2.2 and complement	7.2 ± 0.3	0.7
4	Mixture of cells of groups 2 and 3 at a ratio of 1:1	6.6 ± 0.2	1.3
5	No PETLEs	7.9 ± 0.3	—

a. *L. monocytogenes*-specific PETLEs (3×10^6) or equivalent numbers after treatment were injected intravenously together with 10^5 living *L. monocytogenes*. All experiments were performed with C57BL/6 mice.

b. The values indicate means ± standard deviation of five mice per group.

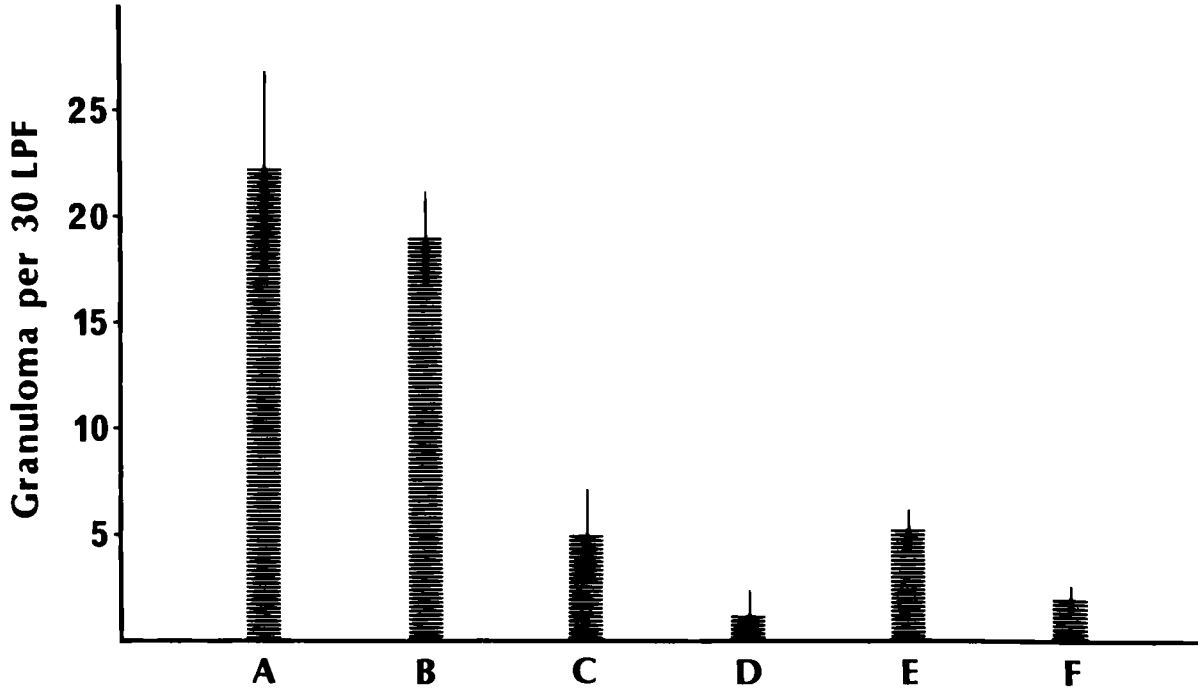

Figure 1. Effect of anti-Ly antisera plus complement treatment on the capacity of *L. monocytogenes*-immune PETLEs to transfer systemic granuloma formation; 5×10^6 unselected PETLEs (A) treated with complement alone (B), or equivalent numbers of anti-Ly-1.2 antiserum plus complement (C), anti-Ly-2.2 antiserum plus complement-treated PETLEs (D), or a mixture of C and D in a ratio of 1:1 (E) were injected intravenously together with 5×10^4 *L. monocytogenes*. Injection of 5×10^4 *L. monocytogenes* alone (F). Mean ± standard deviation of five mice per group.

Table IV. Effect of Anti-Ly 2 and Anti-L3T4 Antisera Plus Complement Treatment on the Capacity of L. monocytogenes-immune Peritoneal Exudate T Lymphocyte-enriched Cells to Transfer Granuloma Formation

Group	*Treatment of specific PETLEs*[a]	*Granulomas*[b] *per 30 LPF*
1	Complement	24.0 ± 6.2
2	Anti-L3T4 and complement	3.7 ± 1.5
3	Anti-Ly-2.2 and complement	1.9 ± 1.2
4	Mixture of cells of groups 2 and 3 at a ratio of 1:1	21.4 ± 4.9
5	No PETLEs	4.3 ± 2.1

Abbreviation: LPF, low power field.

a. *L. monocytogenes*-specific PETLEs (3×10^6) or equivalent numbers after treatment were injected intravenously together with 10^5 living *L. monocytogenes*. All experiments were performed with C 57 BL/6 mice.

b. The values indicate means ± standard deviation of five mice per group.

Table V. Genetic Restriction of Adoptive Transfer of Granuloma Formation

Recipient[a]	*H-2 complex* K I D A J E	*Homology*	*Granulomas per 30 LPF*[b]
C 57B1/6			2.0 ± 1.4
C 57B1/6	b b b b b	All	33.2 ± 8.4
B 10.A (4R)	k k b b b	I-J,I-E,D	2.4 ± 1.7
B 10.A (5R)	b b k k d	K,I-A	30.8 ± 7.3
B 10.MBR	b k k k q	K	36.4 ± 5.7
C 57B1/6			1.8 ± 1.3
C 57B1/6	b b b b b	All	32.7 ± 9.7
B 10.A (4R)	k k b b b	I-J,I-E,D	2.3 ± 1.2
B 6.C-H-2^{bm12}	b . b b b	All except I-A	29.5 ± 6.3
B 6.C-H-2^{bm1}	. b b b b	All except K	2.5 ± 1.3

Abbreviations: PETLEs, peritoneal exudate T lymphocyte-enriched cells; LPF, low power field.

a. 5 × 10^6 *L. monocytogenes*-immune PETLEs were injected i. v. into congenic or mutant recipients which received living *L. monocytogenes* for evaluation of granuloma formation. Data taken from Näher et al. (19).

b. The values indicate mean ± standard deviation of five mice per group.

Näher et al. (19) proposed that macrophage activation and granuloma formation might be phenomena that are based upon different underlying mechanisms and are possibly due to different T cell subpopulations. In order to solve this question, these authors developed a model for the quantitative assessment of adoptive granuloma formation (21).

Listeria-specific T cells from peritoneal exudates of *Listeria*-immune mice were adoptively transferred to normal recipients. The latter were subsequently challenged with *L. monocytogenes*, and the numbers of granulomas in infected livers were determined by light microscopy. Adoptive granuloma formation was abolished when the transferred T cells were subjected to in vitro treatment with either anti-Ly-1, anti-Ly-2, or anti-L3T4 antisera plus complement (Figure 1, Table IV). Thus, granuloma for-

mation, as is the case with protection as a whole, depends on a least two T cell populations, one being L3T4$^+$ Ly-1$^+$2$^-$, the other being L3T4$^-$ Ly-1$^+$2$^+$. When T cells negatively selected for either of these markers were transferred alone, no granulomas were found in challenged cell recipients (22; Sperling et al., manuscript in preparation). Furthermore, employing mice with an isolated genetic defect either at the I-A or at the K region of the MHC, it could be shown that compatibility at the K, but not the I, locus of the major histocompatibility gene complex was necessary and sufficient for the adoptive transfer of granuloma formation (19) (Table V).

Furthermore, the presence of living bacteria in recipients is required for adoptive granuloma formation, whereas in vitro functions of T cells can be observed easily by using heat-killed *Listeria* organisms. We assume that, whereas L3T4 cells can recognize dead listerial antigen, only "living" antigen is seen by *Listeria*-specific T cells of the Ly-2 phenotype (Table VI) (19).

Table VI. Effect of Living and Heat-killed L. monocytogenes Organisms on the Expression of Granuloma Formation in Adoptively Immunized Mice

Number of immune PETLEs transferred	Listeria *antigen*	*Granulomas per 30 LPF*[a]
—	5×10^4 living	2.8 ± 1.6
5×10^6	5×10^4 living	26.4 ± 5.1
—	1×10^5 heat-killed	3.4 ± 2.7
5×10^6	1×10^5 heat-killed	2.8 ± 1.9
—	1×10^6 heat-killed	1.7 ± 2.1
5×10^6	1×10^6 heat-killed	2.4 ± 2.2
—	1×10^7 heat-killed	2.9 ± 1.4
5×10^6	1×10^7 heat-killed	3.7 ± 2.5
—	1×10^8 heat-killed	2.2 ± 1.7
5×10^6	1×10^8 heat-killed	1.9 ± 1.3

Abbreviation: LPF, low power field. *L. monocytogenes*-immune PETLEs were injected i. v. into recipients which received either living or graded doses of heat-killed *L. monocytogenes* for evaluation of granuloma formation (from Näher et al., 19).

a. The values indicate means ± standard deviation of five mice per group.

Table VII. Proportion of Cells within Incipient and Mature Granulomas Reactive with a Given Monoclonal Antibody

	% reactive cells within granulomas	
Surface antigens	*Incipient*[a]	*Mature*[a]
Ly-1	22.3 ± 3.7[b]	41.0 ± 3.7
L3T4	19.3 ± 2.2	39.1 ± 4.3
Ly-2	24.6 ± 3.2	20.2 ± 3.1

Data taken from Näher et al. (22).

a. 60 h (incipient) or 96 h (mature), respectively, after adoptive transfer of 5×10^6 *L. monocytogenes*-immune PETLEs and 5×10^4 *L. monocytogenes*.

b. Means ± standard deviation of five granulomas.

Immunohistology

The role of the two T cell populations has also been scrutinized by immunohistologic analysis of their distribution in granulomatous lesions in livers of infected mice. We were able to show (22) that the fraction of cells in granulomas bearing the L3T4 marker increased from 60 to 96 hours after adoptive transfer of immune T lymphocytes together with living *L. monocytogenes* organisms, whereas the relative amount of Ly-2$^+$ cells remained constant (Table VII). These results indicate a critical role of Ly-2$^+$ cells in the induction phase of granuloma formation.

In Vitro Studies

In vitro studies on *Listeria*-T cell interactions were first performed by Farr et al. (23). An assay developed by these authors permits measurement of T cell proliferation, interleukin 1 (IL-1) (lymphocyte-activating factor) production by activated macrophages, and interleukin 2 (IL-2) (T cell growth factor) production by stimulated T cells. It also allows measurement of the expression of IL-2 receptors.

Interaction of *Listeria* antigen (heat-killed *L. monocytogenes*) in the presence of antigen-presenting normal macrophages with *Listeria*-specific PETLEs yielded results that indicated that antigen-specific proliferation, IL-2 production, and induction of IL-1 production by macrophages all are functions of T cells of phenotype Ly-1$^+$2$^-$ (Table VIII) (10, 24, 25).

Table VIII. Ly Phenotype of T Cell Subsets Involved in T Cell Proliferation and Interleukin Production

Group	*Treatment of PETLs*[a]	*T cell subset*	*Proliferation ([³H]TdR uptake)*[b] *cpm*	*Interleukin activity ([³H]TdR uptake uptake of thymocytes)*[c] *cpm*	*Interleukin 2 activity (% specific lysis of ^{51}Cr-labeled P815)*[d]
1	None	Unselected	38,400	8,500	70
2	C	Unselected	39,900	8,200	63
3	Anti-Thy-1.2 + C	No	320	130	0
4	Anti-Ly-1.2 + C	Ly 1^-23^+	280	180	0
5	Anti-Ly-2.2, 3.2 + C	Ly 1^+23^-	40,100	100	67
6	Mixture of groups 4 and 5	Ly 1^-23^+	44,600	4,100	68
	+	Ly 1^+23^-			
7	No PETLEs	—	1,400	110	66

Abbreviations: PETLEs, peritoneal exudate T lymphocyte-enriched cells; TdR, thymidine.

a. PETLEs were treated with anti-Ly antisera plus C and equivalents of 1×10^6/ml PETLEs were cocultured with normal macrophages and heat-killed *L. monocytogenes*.

b. Proliferative response after 3 days, last 18 hours with 0.1 μC1[³H]TdR (from Kaufmann [24]).

c. Proliferative response of thymocytes in presence of 25% cell-free supernatant after 3 days, last 18 hours with 0.1 μCi [³H]TdR (from Kaufman et al. [25]).

d. Cytotoxic activity of alloreactive cytotoxic T cells after coculture with 50% supernatant. Killer/target ratio 10:1 (from Kaufmann [10]).

Table IX. Macrophage-activating Factor Production by Antisera-treated Listeria-immune Peritoneal Exudate T Lymphocyte-enriched Cells

Group	*Number of* Listeria-*immune PETLEs/well*	*Treatment*	*% specific* ^{51}Cr *release*	*% reduction of* [^{3}H]*TdR incorporation*
1	3×10^5	Nil	59.9 ± 0.1	91.3 ± 2.3
2	1×10^5	Nil	60.9 ± 1.8	57.9 ± 17.8
3	3×10^5	Complement	48.4 ± 1.0	88.2 ± 1.2
4	3×10^5	αLy-1 + Complement	− 2.2 ± 2.7	3.1 ± 1.1
5	3×10^5	αLy-2 + Complement	53.3 ± 7.1	70.2 ± 4.5
6	Recombination of group 4 and 5		65.0 ± 5.4	81.5 ± 7.7

Abbreviations: TdR, thymidine; HKL, heat-killed *Listeria* antigen. Specific ^{51}Cr release and [^{3}H]TdR incorporation by EL_4 tumor cells cocultured with normal peritoneal exudate macrophages (10^6 in 0.8 ml for ^{51}Cr release, 3×10^5 in 0.2 ml for [^{3}H]TdR incorporation) activated by culture supernatants obtained by incubating antisera-treated or untreated *Listeria*-immune PETLEs with 10^6 macrophages and 2×10^6 HKL in a total volume of 0.8 ml or with 3×10^5 macrophages and 4×10^5 HKL in a total volume of 0.2 ml, respectively. Values are means of at least three determinations ± standard deviations (from Sperling et al. [13]).

Table X. Production of Macrophage-activating Factor by Listeria-immune Peritoneal Exudate T Lymphocyte-enriched Cells after Treatment with Anti-L3T4 Antibody and Complement

Group	*No. of* Listeria-*immune PETLEs/ml*	*Treatment*	*% Specific ^{51}Cr release*	*% reduction of [^{3}H]TdR incorporation*
1	1×10^6	Complement	22.8 ± 2.8	71.4 ± 3.5
2	3×10^5	Complement	12.1 ± 4.3	57.4 ± 4.6
3	1×10^6	Anti-L3T4 + complement	−0.7 ± 1.7	9.1 ± 3.5
4	3×10^5	Anti-L3T4 + complement	−2.1 ± 3.5	10.2 ± 5.9

Abbreviations: TdR, thymidine; HKL, heat-killed *Listeria* antigen. Specific ^{51}Cr release and reduction in [^{3}H]TdR incorporation were used to measure the killing of EL_4-tumor cells after coculture with normal peritoneal exudate macrophages (1.5×10^6/ml, total volume 0.2 ml) activated by culture supernatants obtained by incubating *Listeria*-immune PETLEs with macrophages (10^6/ml) and HKL 10^8/ml). Values are means of at least three determinations ± standard deviations.

The in vitro production of macrophage-activating factor (MAF) is another parameter for the in vitro activity of specific T lymphocytes. Hence, macrophage activation is assumed to play an important role in immunity against facultative intracellular bacteria. We have been able to show that MAF production is mediated by Ly 1^+2^-, L3T4$^+$ T lymphocytes (Tables IX and X).

Cloned T Cells

The availability of methods for the cloning of T cells has made it feasible to analyze T cell–macrophage interactions in vitro even more extensively and to determine which T cell population does what in whole animals. Kaufmann and Hahn (26) produced murine T cell clones and Stolpmann et al. (27) produced rat T cell clones, both with specificity to *L. monocytogenes*.

Table XI. Listeria-specific T Cell Proliferation of Rat T Cell Clones in Interleukin 2-containing Conditioned Medium

Clone	*Listeria-specific T cell proliferation*[a] *in the presence of*					
	HKL and accessory cells	*HKB and accessory cells*	*Accessory cells*	*HKL*	*HKB*	*IL-2 only*
	cpm					
RVIIC2	53,783	2,182	2,179	4,638	560	1,634
$R_2$3D6	45,605	6,259	6,946	5,915	1,794	1,722
R30D5	21,397	5,510	2,780	6,861	2,348	2,660

Abbreviations: HKL, heat-killed *L. monocytogenes* organisms; HKB, heat-killed *Brucella abortus* organisms.

a. [^{3}H]TdR uptake tested in triplicates on day 3 of the culture period. In the absence of IL-2, proliferation of T cells was < 1,000 cpm. Standard error of the mean was less than 15%.

Table XII. Phenotypic Characterization of Rat T Cell Clones Using the Monoclonal Antibodies W3/25 (Recognizing the T_{helper} Cell Marker) and MRC OX 8 (Recognizing the $T_{cytotoxic}$ or $T_{suppressor}$ Cell Marker)

Clone	*Phenotype*[a]		
	W3/25 plus ^{125}*I-SAM*	*MRC OX8 plus* ^{125}*I-SAM*	^{125}*I-SAM only*
	cpm/5 × 10^5 cells		
RVIIC2	51,186	3,605	3,690
$R_2$3D6	40,797	1,260	1,976
R30D5	34,763	45,285	3,757

a. Binding of ^{125}I-sheep anti-mouse Ig (^{125}I-SAM) to cells pretreated with the respective monoclonal antibody.

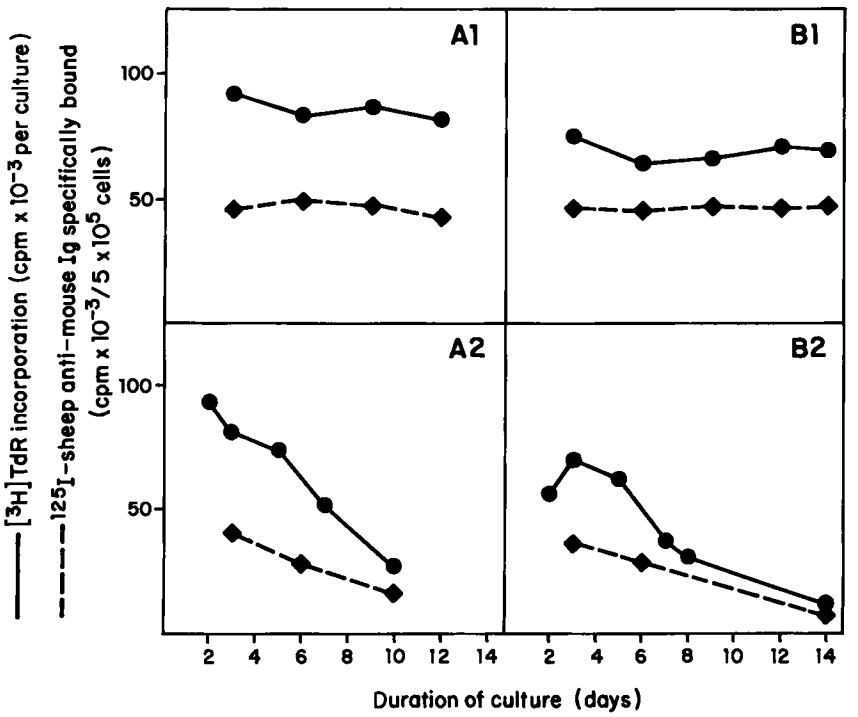

Figure 2. Kinetics of IL-2 receptor expression (♦) and proliferation (●) of *Listeria*-specific rat T cell clones RVIIC2 (A) and $R_2$3D6 (B). Cells were stimulated with heat-killed *Listeria* antigen every 3 days (A1 and B1) or cultured in the absence of additional antigen (A2 and B2). For details see text. The standard error of the mean was less than 15%. Figure was adapted from Stolpmann et al. (27).

Murine T Cell Clones

Murine T cell clones were produced using the method of Sredni et al. (28) and were of the L3T4$^+$ Ly-1$^+$2$^-$ phenotype (13). Such cloned cells were able to mediate adoptively both DTH and a certain degree of protection. In addition, *Listeria*-specific cloned T cells were able to produce inteferon-gamma (11) and MAF (13). Likewise, when supernatants from antigen-stimulated, cloned T cells were injected into normal mice, the latter exhibited some degree of protection after subsequent challenge with *L. monocytogenes*, most likely because of systemic activation of macrophages.

Rat T Cell Clones

The results obtained with cloned *L. monocytogenes*-immune T lymphocytes were confirmed and extended by Stolpmann et al. (27) using LEW rats. Splenic T lymphocytes from rats immunized with *L. monocytogenes*

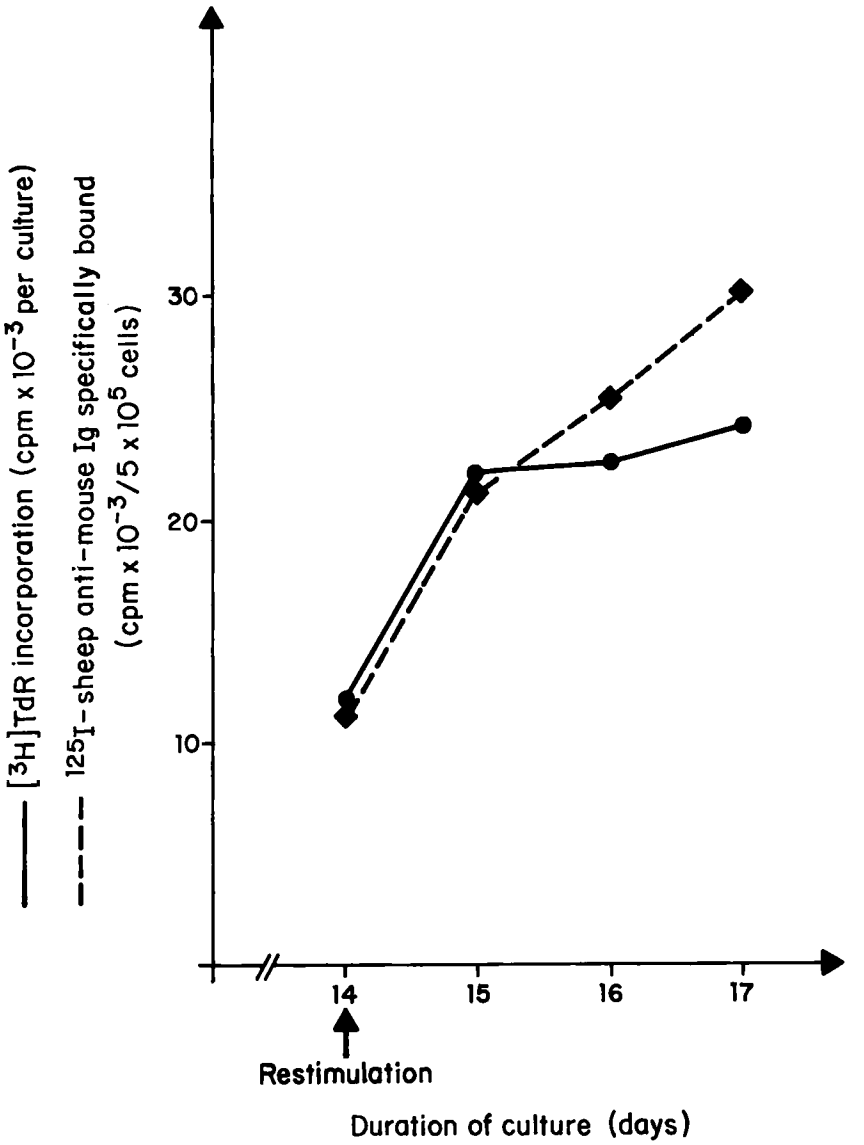

Figure 3. Antigen-dependent reexpression of IL-2 receptors (♦) and sensitivity to IL-2 action (●) of the clone $R_2$3D6. Cells that lost IL-2 receptors during 14 days of culture were restimulated with heat-killed *Listeria* antigen. Figure was adapted from Stolpmann et al. (27).

were cloned by the limiting-dilution technique (29). Cloned rat T cells were *Listeria*-specific, and their proliferation depended on class II-restricted antigen presentation by accessory cells (Table XI). Three of the clones

Table XIII. Production of Migration Inhibition Factor and Macrophage-activating Factor by Listeria-specific Rat T Cell Clones

Clone	*% inhibition of migration*	*% reduction of [^{3}H]TdR incorporation*
RVIIC2	15.5 ± 2.6	72.9 ± 1.0
$R_2$3D6	25.6 ± 4.6	88.8 ± 3.3
R30D5	43.0 ± 10.1	86.1 ± 5.2

Abbreviation: TdR, thymidine.

Table XIV. Capacity of Listeria-specific Rat T Cell Clones to Mediate the Systemic Adoptive Protection against L. monocytogenes infection

T cell clone[a]	*Log units of protection*[b]
1. RVIIC2	0.18 ± 0.08
2. $R_2$3D6	0.92 ± 0.11
3. R30D5	1.00 ± 0.20

a. *L. monocytogenes*-specific cloned T cells (2×10^6) were injected intravenously together with 5×10^6 living *L. monocytogenes* into syngeneic rats.

b. Values indicate means ± standard deviation of five rats per group.

obtained were scrutinized for their biological activities. As demonstrated by their reactivity to the monoclonal antibodies W 3/25 and MRC OX 8, two of the clones were of the helper T cell phenotype, whereas one showed reactivity with both antibodies (Table XII). Cloned cell proliferation depended on exposure to heat-killed *Listeria* antigen. When antigen was omitted from the system, cell growth subsided over time, and cells finally ceased to grow. By the use of the monoclonal antibody ART-18, which recognizes the IL-2 receptor (a gift from Professor Diamantstein), it was shown that cessation of growth was accompanied by the disappearance of IL-2 receptors from the cell surface (Figure 2). Addition of heat-killed *Listeria* antigen to the culture resulted in reexpression of IL-2 receptors and concomitant resumption of proliferation (Figure 3).

The T cell clones mentioned were screened for multiple biological activi-

ties. They were not capable of IL-2 production, but were able to produce MAF and migraton inhibition factor (MIF) in different quantities (Table XIII). Two of them also were capable of protecting nonimmune rats against systemic infection with *L. monocytogenes* (Table XIV), albeit to a limited degree when compared with *Listeria*-immune PETLEs. They did not induce, however, significant granuloma formation. Protective capacity was positively correlated with the quantity of MAF and MIF produced, but did not depend on the serologic phenotype of the clone, inasmuch as both the "pure" helper clone and the "mixed" clone, which expressed both the helper and the suppressor phenotype, acted basically in a similar fashion.

Conclusions

From the above experiments, it is concluded that both macrophage activation and granuloma formation contribute to the effective buildup of an antibacterial defense. Macrophage activation, on the one hand, is a function of H-2 I-A-restricted Ly-1$^+$2$^-$ L3T4$^+$ T cells via secretion of interferon-gamma. For granuloma formation, on the other hand, additional functions performed by Ly 1$^+$2$^+$ L3T4$^-$ T cells are necessary. Ly 1$^+$2$^+$ L3T4$^-$ T cells are restricted by H-2 K, whereas macrophage-activating, Ly-1$^+$2$^-$ L3T4$^+$ T cells are restricted by H-2 I-A. Ly-1$^+$2$^+$ L3T4$^-$ T cells, in order to function properly, must recognize living antigen, whereas for the Ly 1$^+$2$^-$ L3T4$^+$ cell-dependent functions, dead antigen suffices.

Summary

This chapter has summarized recent studies concerning the requirements for immunity against the facultative intracellular pathogen *Listeria monocytogenes*. By employing monoclonal antibodies against the L3T4 and the Ly-2 antigen, it has been demonstrated that adoptive transfer of protection and accelerated granuloma formation in infection with *L. monocytogenes* are due to the T helper as well as the T cytotoxic/suppressor subsets. Both T cell populations can be found in granulomatous lesions by means of an immunohistochemical staining procedure. Experiments with anti-Ly-1 antibody reveal that both functionally active T cell populations in this system in addition are Ly-1 positive.

In contrast to the in vivo findings, in vitro activities such as proliferation or the production of IL 2, MAF, and MIF, are exclusively performed by Ly-1$^+$2$^-$ L3T4$^+$ T cells. These findings could be confirmed using *Listeria*-specific T helper cell clones both of mouse and rat origin. These cells,

in spite of being highly active in such in vitro functions as antigen-specific proliferation and production of MAF and MIF, were poorly protective when injected systemically into syngeneic recipients and not capable of inducing granuloma formation.

The expression of the IL-2 receptor on cloned *Listeria*-specific T helper cells of rat origin was analyzed with the monoclonal antireceptor antibody ART 18. Receptor expression was found to depend on the presentation of specific antigen to the T lymphocytes.

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Human Leprosy: Defects in Cell-mediated Immunity

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Entity of the Disease

Leprosy or Hansen's disease is a chronic granulomatous disease caused by the obligate intracellular parasite *Mycobacterium leprae*. Because of the association of leprosy with the loss of digits and mutilating skin lesions, the disease has always generated an unusually strong emotional response. The advent of antimicrobial therapy has changed both the clinical course of the disease and the practice of segregation of patients in isolated communities. However, leprosy continues to infect millions of people leading to a lifetime of social ostracism and constant chemotherapy. It is calculated at present that between 15 and 20 $\times$ 10^6 cases exist worldwide, and these are largely found in subtropical climes.

For the medical scientist leprosy represents a particularly chronic disease, largely localized to the dermis in which host-parasite interaction is controlled by the effector cells generated by cell-mediated reactions. Neither the reservoir of organisms in the environment nor the mode of transmission to humans is clear. Recent observations indicate that a small percentage of armadillos in the southwestern United States contain an organism indistinguishable from *M. leprae* in humans (1). Whether these animals, which are susceptible hosts, can transmit the disease or are in themselves the hosts of human bacilli is still unknown.

Spectrum and Polar Forms

The clinical manifestations of leprosy vary tremendously depending upon the immune status of the host (2). In the more severe lepromatous disease, leprosy bacilli multiply to high levels in the cells of the dermis. Organisms can be discerned in macrophages, Schwann cells, endothelial cells of small blood vessels, and occasionally fibroblasts. Associated with the huge numbers of bacilli are a variety of skin lesions and the leonine facies of patients with generalized disease of periorbital and nasal regions. At the other end of the spectrum, or at the tuberculoid pole, only rare

bacilli are seen and the host is capable of controlling bacillary multiplication. Many intermediate forms exist. Often as a result of chemotherapy or undetermined fluctuations in the patient's immune status, the disease may either increase or decrease in severity.

Organism of the Disease

Mycobacterium leprae is an acid-fast bacillus with an unusually slow rate of growth (3). Many attempts at culturing it on artificial media have met with failure, and until the last few decades no method was available for its propagation outside of the human body. Two discoveries have changed this situation and have led to striking advances in our knowledge of the organism and host defense mechanisms. In the first, the mouse foot pad was found to be a localized site that supported bacterial replication (4). This site is commonly employed for the evaluation of organism viability and drug sensitivity. Such assays, however, still require a prolonged period of 6 months or more to be completed. A second major advance came with the observations that the nine-banded armadillo could be infected with human strains of *M. leprae* and that the organism would grow to very high numbers in the liver and other viscera (5). Again, a period of 9 months to a year is required for maturation of the lesions. Nevertheless, armadillo spleens and livers have proven a valuable source for large numbers of pure, viable bacilli and have facilitated the chemical and metabolic characterization of important lipid and protein constituents. It is thought that the relatively low body temperature of the armadillo (31°C) as well as that of the mouse foot pad contribute to the ability of the organism to replicate.

Purified bacilli from armadillo livers have served as the starting point for the identification of *M. leprae* components. A variety of polypeptides can be displayed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Some of these contain *M. leprae*-specific epitopes, but they all contain antigenic sites that are shared by other mycobacteria. For the most part, their functions and localization in or on the bacterium are poorly understood. Monoclonal antibodies have now been prepared against many of the more common species and serve as useful diagnostic and preparative reagents (6, 7).

Recently, complementary DNA libraries of *M. leprae* have been prepared and a number of *M. leprae* polypeptides are now being expressed by *Escherichia coli* (8) and should be available in large amounts in the next few years.

Through the efforts of Patrick Brennan and his colleagues (9), several

M. leprae-specific glycolipids have been identified and chemically characterized. These compounds appear to have, in part, a surface or even extrabacillary localization. The antigenic determinants are contained in terminal trisaccharides composed of methylated glucose and rhamnose moieties. Sera from patients with leprosy contain antibodies against these compounds. This serves as the basis for a sensitive method of detecting overt infection and household contact (10).

Histopathology and the Disease Process

Lepromatous Leprosy

The lesions of lepromatous patients are characterized by the presence of dermal accumulations of heavily infected macrophages and a sparse infiltrate of lymphoid cells. Parasitized macrophages are present in almost all regions of the skin and often contain large aggregrates or globi of acid-fast bacilli. Lymphocytes and macrophages are not present in any organized, granulomatous association and are contained within bundles of collagen fibers. Infected macrophages often have a "foamy" appearance on sections (11).

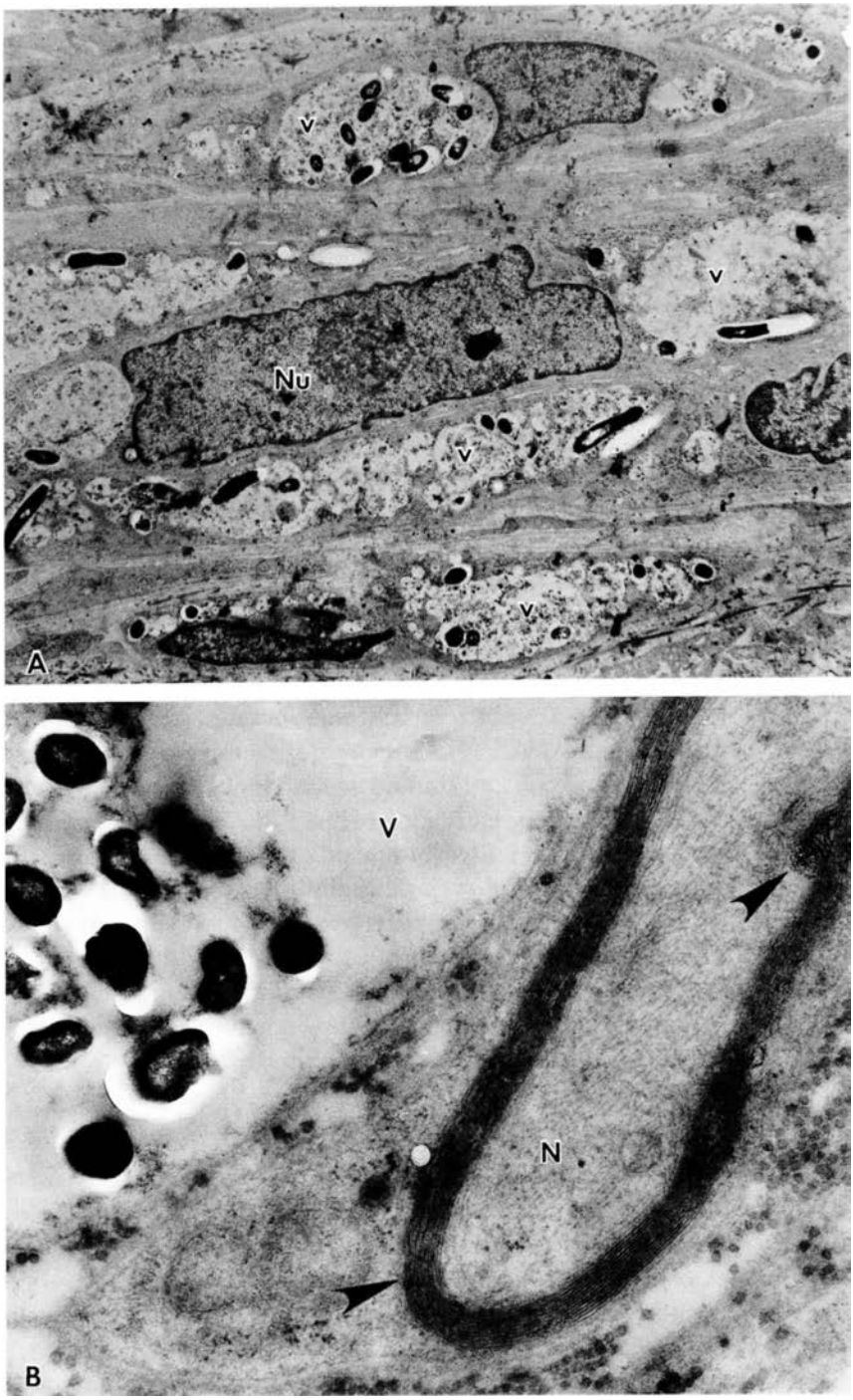
At the level of transmission electron microscopy, *M. leprae* is seen as an osmiophilic bacillus within membrane-bounded phagolysosomes of macrophages (Figure 1 A) (12). The organisms are embedded in an extensive amorphous matrix and contain around them a capsulelike halo that probably results from the extraction of lipids during tissue processing (13). Both intact and fragmented organisms are present in untreated patients, and the number of disrupted bacilli increases with the initiation of chemotherapy. Acid phosphatase reaction product is present within the endosome, suggesting the fusion of phagosome and lysosome (14).

Other than macrophages the most commonly infected cell is the Schwann cell surrounding peripheral nerves (Figure 1 B). Schwann cells often have a bacterial load similar to macrophages.

Tuberculoid Leprosy

As one approaches the tuberculoid pole the histologic picture becomes strikingly different from that of lepromatous disease. Bacilli become fewer in number and the lymphocytic infiltrate increases in size and organization. In fact, acid-fast bacilli and their remnants are scarce at the tuberculoid pole, and organized granulomas with multinucleated giant cells and cuffs of lymphocytes are common (Figure 2).

At the level of electron microscopy, organisms when present are seen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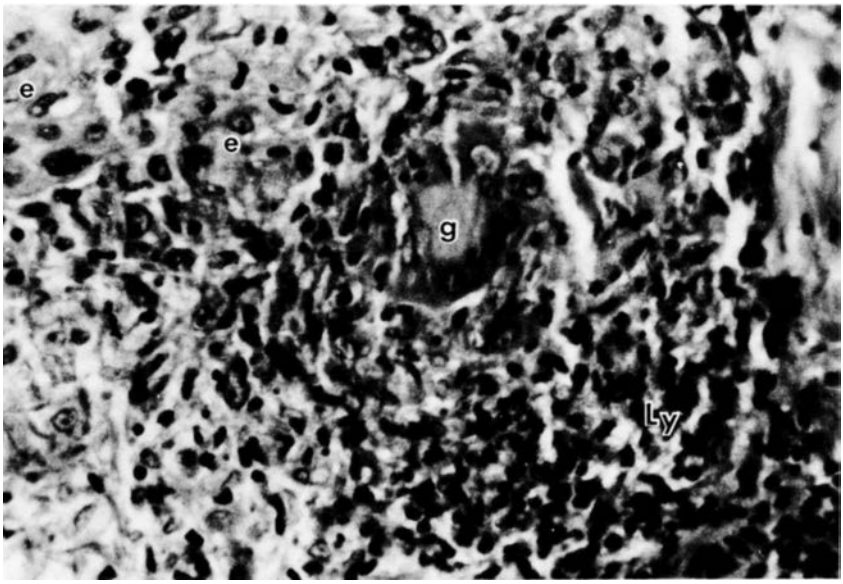

Figure 2. Histologic section through a tuberculoid leprosy lesion. Tuberculoid granuloma containing epithelioid cells (*e*), a large multinucleated Langhans giant cells (*g*), and many lymphocytes (*Ly*). Hematoxylin and eosin; ×389.

within phagolysosomes of the epithelioid cells. These cells have the rough endoplasmic reticulum of secretory cells (Figure 3 A and B). Of particular note are unusual lymphocytes with extensive membrane projections (Figure 3 C). Close to these lymphocytes are areas of cell death (Figure 3 D). Possibly these lymphocytes are cytotoxic and kill macrophages.

Immunopathology of Lepromatous and Tuberculoid Disease

Until the advent of monoclonal antibodies little was known about the nature of the cell types that accumulated in the cutaneous and parenchymatous lesions of humans and animals. However, once these reagents became available and sensitive immunocytochemical and fluorescent methods were developed, the identification of many of the subsets of cir-

Figure 1. Transmission electron micrographs of cutaneous infiltrates from a patient with lepromatous leprosy. (A) The infiltrating cells consist mostly of parasitized foam macrophages, embedded in collagen. The cells have a large nucleus (*Nu*) and many electron-lucent vacuoles (v) containing osmiophilic *M. leprae*; ×6,600. (B) *Mycobacterium leprae* organisms are found in an intracellular vacuole (v) of a Schwann cells. First signs of demyelination of the axon are observed (*arrowheads*); neuron (N); ×27,000.

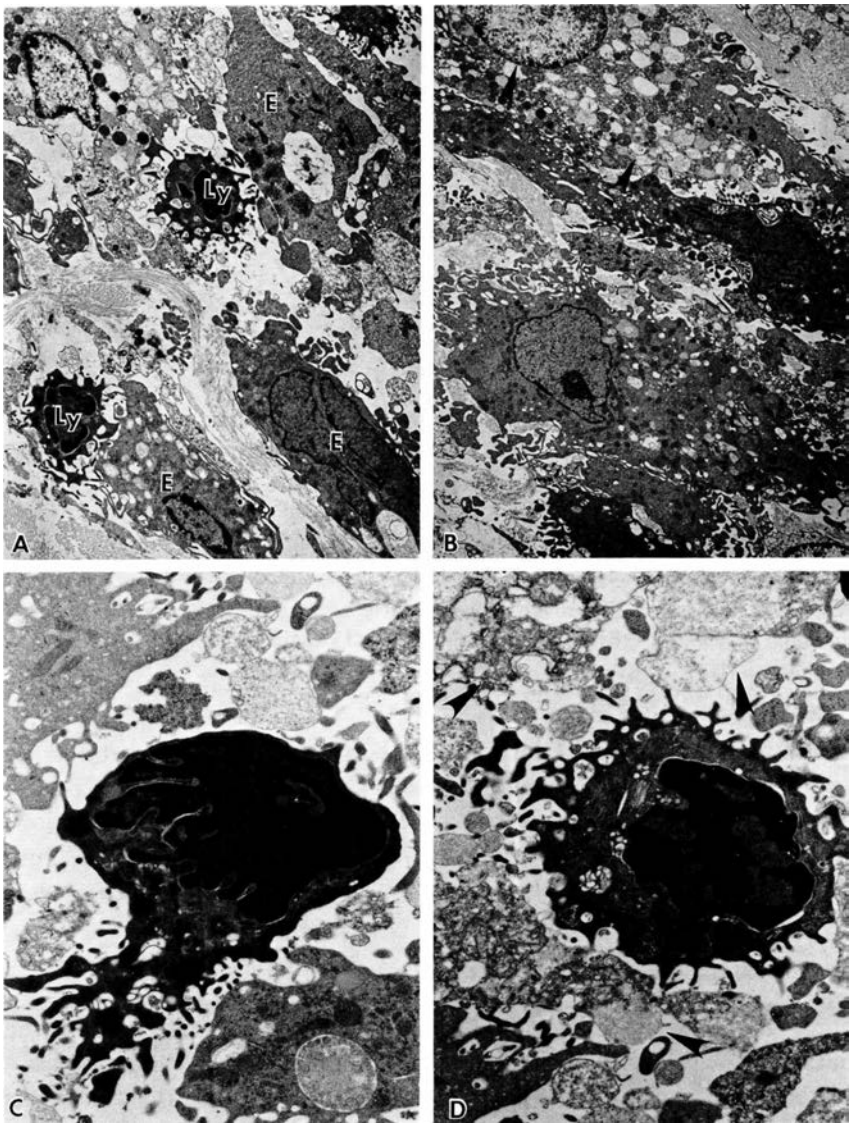

Figure 3. Transmission electron micrographs of cutaneous infiltrates from a patient with tuberculoid leprosy. (A) The granuloma consists mostly of epithelioid cells (*E*) and lymphocytes (*Ly*), embedded in a loose collagen matrix; ×3,000. (B) The epithelioid cells of the lesion are rich in organelles. Many are damaged or dead with granular cytoplasm and nucleus (arrowheads); ×2,500. (C) The lymphocytes of the lesion are often very irregular in shape with extensive cytoplasmic extensions; ×7,000. (D) The lymphocyte extensions are often associated with dead epithelioid cell fragments (*arrowheads*); ×7,000.

culating cells could be achieved. This led to interesting observations that characterized the cells accumulating in the cutaneous lesions of leprosy (15–18). Patients with lepromatous disease demonstrated the presence of Ia^+ macrophages and small numbers of scattered T cells. The vast majority of the T cells were of the Leu-2a/OKT8 (suppressor, cytotoxic) subset of T lymphocytes. Only rare Leu-3a/OKT4 (helper) T cells were present, and the T_4/T_8 ratios were considerably less than unity. As one progressed towards the tuberculoid pole, the number of T cells increased and many of these were of the helper phenotype. This was associated with the granulomatous organization of the lesion (data presented in Table I).

Although lepromatous lesions are essentially devoid of helper T cells, normal numbers circulate in the peripheral blood. The lack of accumulation of Leu-3a/OKT4 helper cells suggests either a lack of a signal for emigration, inhibition of emigration, or a heightened turnover of these cells in the tissues. In either case it points up our ignorance concerning lymphocyte emigration into local lesions and the factors that control the movement of subsets.

T Cell Function in Leprosy

Some of the earliest immunologic distinctions between lepromatous and tuberculoid leprosy resulted from the response of patients to skin testing with *M. leprae* or its products (19). Delayed sensitivity, expressed as local induration, was prominent in the tuberculoid patients and absent in lepromatous disease. However, when tested with other antigens that were immunologically unrelated to *M. leprae*, lepromatous patients demonstrated positive skin tests, which on occasion were more subdued than in tuberculoids or controls (20).

From this type of data and the subsequent in vitro testing of T cell responsiveness, it is generally considered that lepromatous leprosy patients have an antigen-specific defect in cell-mediated immunity, but are capable of responding to other antigens. The exposure of peripheral blood T cells obtained from lepromatous patients to *M. leprae* antigen leads to a poor or absent incorporation of tritiated thymidine, whereas cells from tuberculoid patients respond vigorously (21). In contrast, polyclonal T cell activators such as concanavalin A stimulate tritiated thymidine incorporation to a similar extent in both forms of the disease (22, 23).

The lack of thymidine incorporation by lymphocytes from lepromatous leprosy patients in response to *M. leprae* is also reflected in their lack of production of lymphokines in response to this stimulus. Neither interferon-gamma (IFN-γ) nor the T cell growth factor interleukin 2 are

Table I. Distribution of Leukocytes in Cutaneous Lesions of Untreated Leprosy Patients

Leprosy diagnosis	*Number of patients*	*Percent total cells*			*Ratio*
			T cell[b]		
		Monocytes[a]	*OKT4*	*OKT8*	*OKT4/OKT8*[b]
Tuberculoid	9	25–50	17 ± 4	14 ± 5	1.35 ± 0.44
Borderline tuberculoid	8	30–60	16 ± 7	14 ± 6	1.11 ± 0.49
Mid borderline	3	30–50	25 ± 6	20 ± 4	1.25 ± 0.31
Borderline lepromatous	7	50–70	10 ± 8	17 ± 6	0.48 ± 0.30
Lepromatous	10	55–80	2 ± 1	18 ± 3	0.19 ± 0.15

The results are the mean of those reported by van Voorhis et al. (15) and by Sarno et al. (17).

a. Range.

b. Mean ± standard deviation.

secreted into the environment. However, cells from tuberculoid leprosy patients make adequate amounts (23–25).

The specific lack of responsiveness of lepromatous patients' T cells to *M. leprae* has been explained in a variety of ways (reviewed in Reference 16). Some consider its association with the genetic background of the patient. In other studies lepromatous leprosy patients have been shown to lack or have reduced numbers of circulating T lymphocytes capable of responding to *M. leprae* by proliferation or IFN-γ production (21, 23, 24). This T cell defect is specific for *M. leprae* and is manifested as both the nonresponsive and hyporesponsive states. Suppressor effects expressed by both T cells and monocyte/macrophages have been implicated, and antigens of *M. leprae* such as phenolic glycolipids are reported to depress blastogenesis (27). The depletion of monocytes and $OKT8^+$ (suppressor) T cells from the cultures did not restore responsiveness to *M. leprae* in nonresponder patients, but a nonspecific enhancement of proliferation was observed in monocyte-free cultures from patients that do respond to *M. leprae* (23). Thus, in our hands, the defect in lepromatous nonresponders does not appear to result from a simple suppression by monocytes. In no case are we assured of a comprehensive explanation that encompasses all the clinical states. However, recent increases in our knowledge of the accessory cells, T cell antigen receptors, and secretory products offer new points of attack in understanding this fascinating situation.

Effector Component of Leprosy — Monocytes and Macrophages

The previously described defects in T cell responsiveness in lepromatous patients fit into our understanding of monocyte–macrophage activation and the destruction of intracellular microbes. Based upon an extensive series of investigations, it appears quite certain that lymphokines activate mononuclear phagocytes to take on an enhanced microbicidal capacity. This information was largely obtained from studies with mouse peritoneal macrophages in which the resident or "resting" cell served as a permissive host for large parasites such as *Trypanosoma cruzi* (28), *Toxoplasma gondii* (29), and *Leishmania* species (30). Such obligate intracellular organisms could be readily screened at the level of light microscopy and enumerated. When peritoneal cells were activated in vivo by means of a *Listeria* or Calmette-Guerin bacillus infection or in vitro with crude concanavalin A-stimulated T cell supernatant fluids, the cells acquired the ability to destroy these organisms.

A major mechanism of killing was gradually ascertained with the above models and with the realization that mononuclear phagocytes had the

ability to reduce molecular oxygen to toxic oxygen radicals (31, 32). The two major products were superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), whereas myeloperoxidase was present only in freshly isolated blood monocytes. Before long it became possible to compare the state of intracellular killing with the producton of oxygen species and these were found to be well correlated. More recently, it was found that the macrophage-activating factor of lymphocyte supernatant fluids was in fact gamma- or immune interferon (33), which could stimulate both H_2O_2 formation and intracellular killing.

The nature of this macrophage effector system was next examined in the blood monocytes of patient with lepromatous and tuberculoid leprosy. Monocytes from lepromatous patients had a somewhat reduced ability to release H_2O_2 when stimulated with the soluble trigger phorbol myristate acetate (Nathan et al., manuscript submitted for publication). They could, however, respond to IFN-γ and raise the output of reactive oxygen intermediate into the normal range (Kaplan et al., manuscript submitted for publication). In addition, the exposure of monocytes from lepromatous patients to IFN-γ-containing lymphokines led to their enhanced destruction of another obligate intracellular bacterium, *Legionella pneumophila* (22). Similar experiments have not yet been performed with viable *M. leprae* nor with dermal macrophages because of technical difficulties. The ability of the leprosy bacillus to replicate within the fused phagolysosomes of macrophages from lepromatous patients suggests that inhibition of fusion is not the primary defect in these patients.

Although monocytes from patients with lepromatous leprosy are responsive to lymphokines, T cell production of activating factors is deficient (22). Although T cells from tuberculoid patients or control subjects could generate a macrophage-activating factor with, respectively, *M. leprae* antigens or lectin, the lepromatous leprosy patients' T cells failed to produce such a factor in response to *M. leprae*—that is, failed to produce a factor that led to the killing of *L. pneumophila* (Figure 4). This was, in fact, well correlated with inability of the T cells to produce IFN-γ upon exposure to *M. leprae* (Figure 5). Extrapolating to the local cutaneous lesion, we suspect that both the absence of appreciable markers of T_4 helper cells in the dermis and their unresponsiveness to *M. leprae* result in the absence of IFN-γ and the lack of macrophage activation. This in turn leads to the slow but inexorable replication of *M. leprae* in permissive macrophages, the production of huge amounts of antigen (10^9 bacilli/g), and the subsequent sequelae of long-standing Hansen's disease. In contrast, the tuberculoid patient focuses helper cells into the lesions and responds to *M. leprae*. Interleukin 2 is secreted, and T cells expand

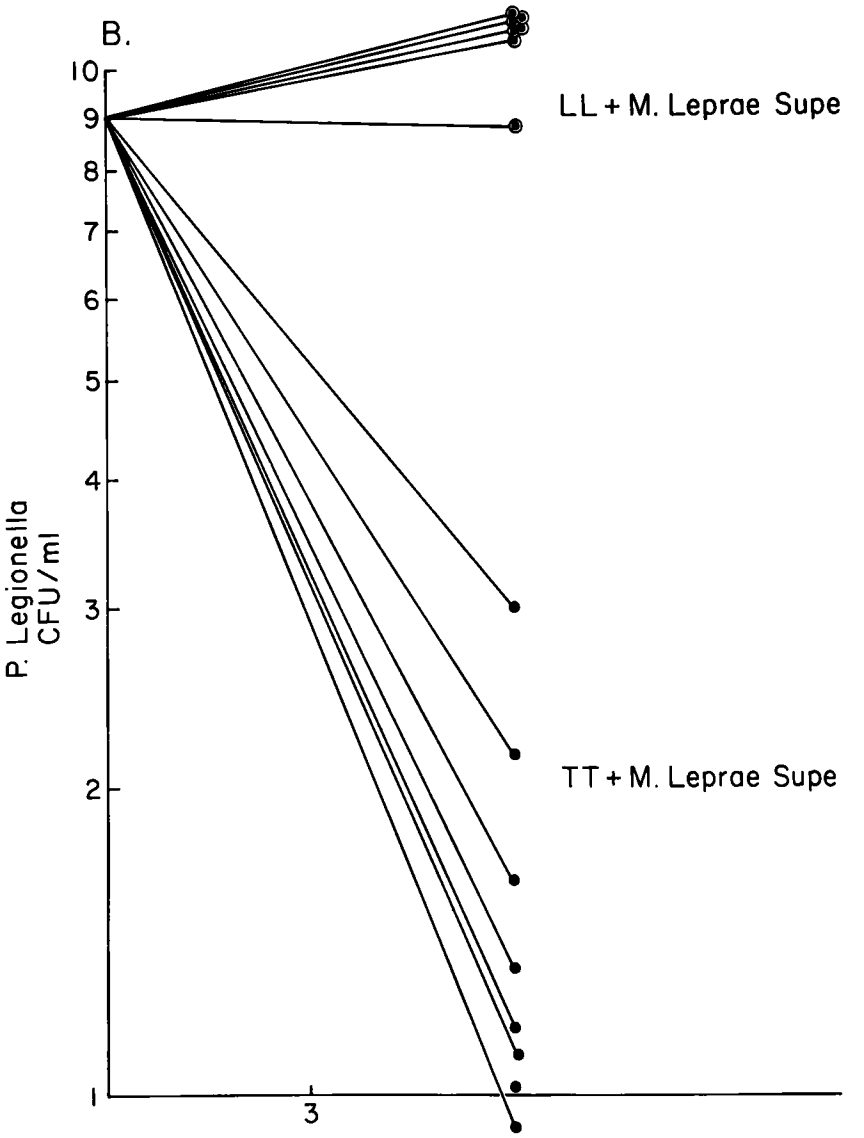

Figure 4. The supernatant activity of *M. leprae*-stimulated peripheral blood mononuclear cells from patients with lepromatous (LL) and tuberculoid (TT) leprosy in stimulating normal monocytes to control the growth of intracellular *Pneumophila legionella.* T cells from patients with lepromatous leprosy fail to generate monocyte-activating factors.

in number and generate macrophage activating IFN-γ. The resulting increase in the capacity to produce peroxide or other reactive oxygen intermediates inactivates intracellular *M. leprae.* This tentative scenario fits

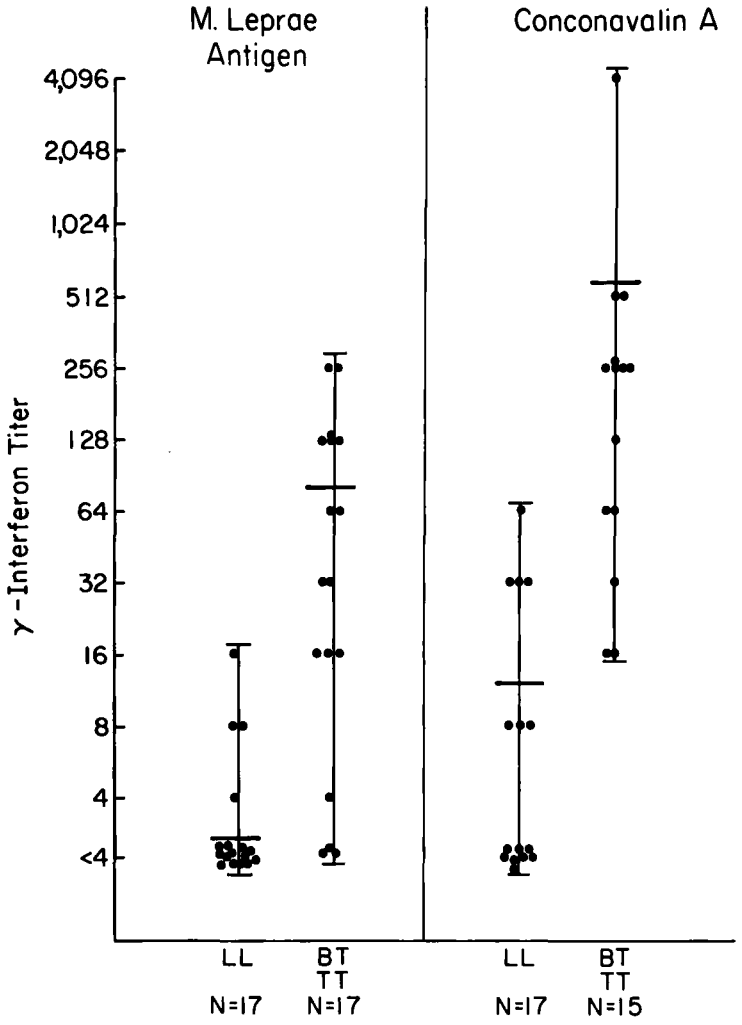

Figure 5. The ability of *M. leprae* antigens and concanavalin A to stimulate the production of γ-IFN by peripheral blood mononuclear cells from patients with tuberculoid (BT and TT) and lepromatous (LL) leprosy. T cells from lepromatous leprosy patients fail to secrete the macrophage-activating factor γ-IFN in response to *M. leprae* antigens.

with many of the existing facts and may be more directly examined in the future.

Mycobacterium leprae has been shown to induce the release of H_2O_2 secretion from human monocytes. In contrast to the response with other microorganisms (Nathan, unpublished observations), the effect of *M. leprae* was markedly delayed in onset, prolonged in duration, and dependent

on high bacteria/monocyte ratios (Nathan et al., manuscript submitted for publication). It is of some interest that *M. leprae* does not contain catalase, a scavenger of peroxide, and is killed by H_2O_2 as measured by its viability after injection into the mouse foot pad (35, 36).

Tuberculin Reactions in the Dermal Lesions of Leprosy

The absence of T helper cells in cutaneous lesions of lepromatous patients focused our attention on the ability of the dermal lesions to provide the necessary milieu for T_4 emigration, persistence, and function. For this purpose we chose to generate tuberculin reactions in the skin of lepromatous patients and examine the nature of the emigrating cells and response of the lesions (Table II). A portion of lepromatous patients responded with areas of induration 10–30 mm in diameter, and these as well as adjacent skin were biopsied at 72 hours after antigen administration. Analysis with monoclonal antibodies and immunoperoxidase staining revealed the influx of large number of T cells of *both* T_4 and T_8 subsets along with blood-borne monocytes. In addition, the $T6^+$, Ia^+ Langerhans cells of the epidermis were now found in the dermis in moderate numbers (manuscript in preparation). These cells are potent accessory cells for T cell-mediated reactions and have the properties of mouse splenic dendritic cells and human blood dendritic cells (37, 38). At the level of electron microscopy, significant numbers of "foamy" bacilli-laden macrophages

Table II. Purified Protein Derivative Responsiveness in Leprosy Patients and in Nonleprosy Controls

Leprosy diagnosis	*Number of patients*	*Responder numbers (percent)*
Lepromatous	25	14 (56)
Borderline lepromatous	10	8 (80)
Borderline tuberculoid and tuberculoid	55	44 (80)
Control	50	34 (68)

The delayed type hypersensitivity reaction to a single injection of 5 U purified protein derivative administered intradermally into lepromatous leprosy, borderline lepromatous leprosy, borderline tuberculoid and tuberculoid leprosy, and nonleprosy control was evaluated. The numbers and percent responders are shown.

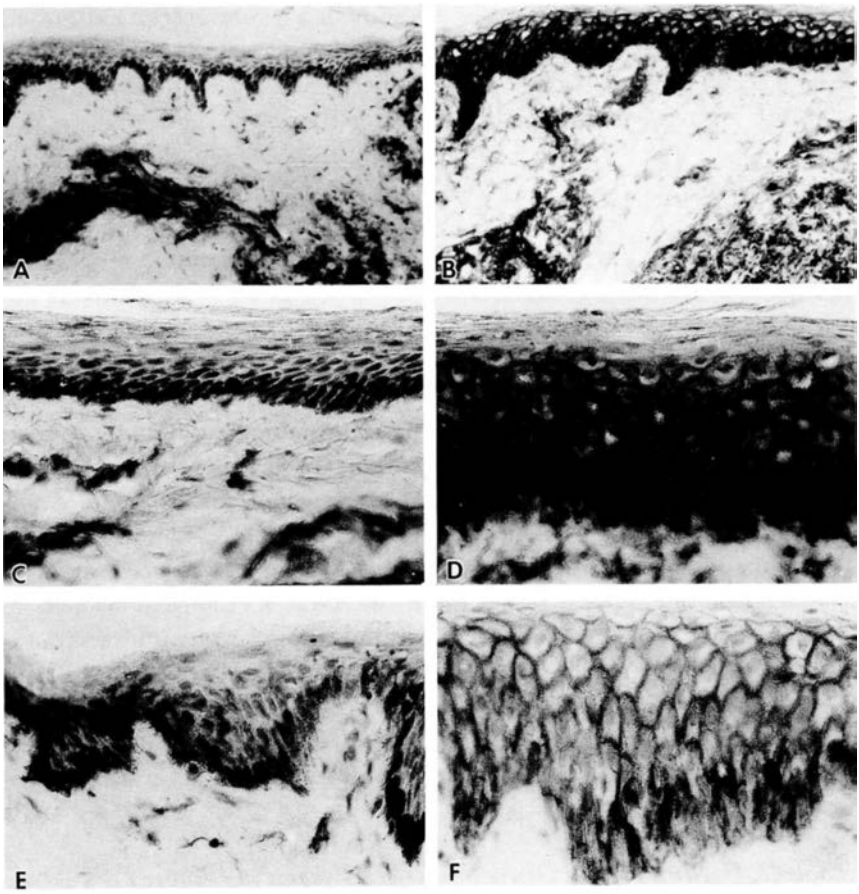

Figure 6. The expression of keratinocyte Ia in response to intradermal tuberculin reactions. Photomicrographs of anti-Ia (9.3F10) monoclonal staining of the epidermal cells of uninjected leprosy lesions (A, C, E) and tuberculin-responsive injected lesions (B, D, F) of leprosy patients. The tuberculin response is accompanied by the induction of keratinocyte Ia as well as keratinocyte hyperplasia and hypertrophy. Keratinocyte Ia staining is observed only above the delayed-type hypersensitivity reaction and not in adjacent skin. The dark stain in the basal layer of the uninjected sites is melanin. (A and B, ×107; C–F, ×266)

had been disrupted and extracellular bacilli were seen. The nature of this cytotoxic reaction is currently under study.

One of the most striking changes occurred in the epidermis overlying and restricted to the delayed reaction to purified protein derivative (39). Normally, the skin (lower back) of lepromatous patients is thin with flattened keratinocytes. However, over the course of the purified protein

derivative reaction, the epidermis was thickened (about twofold) with enlarged, more spherical keratinocytes, all of which expressed Ia antigen on their cell bordes (Figure 6, Table III). Cell counts revealed an approximate doubling of cells (layers) with occasional mitotic figures. This suggests the generation of epidermal growth factor(s) by the cells of the delayed reaction and a rapid epidermal response. Similar histologic findings were made when the naturally occurring skin lesions of tuberculoid leprosy and cutaneous leishmaniasis were examined (Table IV, Figure 7). In each case the epidermis was thickened and the cells were strongly Ia positive—whereas the dermis contained a mixture of T_4 and T_8 subsets and macrophages. In contrast, the skin lesions of lepromatous patients were uniformly negative for epidermal Ia and thickening. This again suggests a role for the cells contained in delayed reactions in influencing the state of the epidermis.

Some suggestion as to the nature of the responsible factor(s) has come from simultaneous studies of the effect of recombinant, human IFN-γ introduced into the skin of patients with lepromatous disease (Nathan et al., manuscript submitted for publication). This pure lymphokine when

Table III. Induration, Epidermal Thickening and Keratinocyte Ia Expression in Response to Purified Protein Derivative in the Lepromatous Leprosy Patient

Patients	*No. tested*	*Induration (median)*	*Keratinocyte Ia*			*Mean thickness ratio (E/C)*	*Mean cell layers*	
			+	+/−	−		*C*	*E*
		mm						
Responders	22	10–38 (20)	20	2	0	1.8 ± 0.4	4.4	7.5
Nonresponders	6	< 10 (0)	1	2	3	1.0 ± 0.2	4.6	5.1

Lepromatous and borderline lepromatous leprosy patients were tested for their response to a single injection of 5 U purified protein derivative. The induration was measured in millimeters. Keratinocyte Ia was evaluated and expressed as follows: +, all keratinocytes staining; +/−, foci of staining keratinocytes; −, no keratinocyte staining. Epidermal thickness in uninjected leprosy lesions (C) and purified protein derivative-injected lesions (E) were measured. Results are expressed as the mean of ratio of thickness of the purified protein derivative lesion over the thickness of the uninjected lesion (E/C) ± standard deviation.

Table IV. Keratinocyte Ia in Lesions of Leprosy Patients[a]

Leprosy diagnosis	*Number of patients*	*Incidence of keratinocyte Ia*		
		+	+ / −	−
Lepromatous	9	0	0	9
Broderline lepromatous	6	1	0	5
Borderline tuberculoid	6	3	2	1
Tuberculoid	4	2	2	0

+ , all keratinocytes staining; + / − , foci of staining cells; − , no keratinocytes staining.

a. All patients tested were untreated at the time of biopsy.

placed into the dermis via a jet gun (Medajet) at 1- or 10-μg doses (three times) also has the unusual effect of inducing the expression of Ia antigen on epidermal keratinocytes and increasing skin thickness. Whether it does this directly by an action on the keratinocyte or via another cell type is unclear. In addition, it leads to a significant accumulation of T cells and monocytes in the existing lepromatous infiltrate. This indicates that one can mount a cell-mediated reaction in the lepromatous lesions and that the cells of these lesions are capable of responding to IFN-γ.

Influence of γ-Interferon, and the Role of Nonleukocytes in Cell-mediated Reactions

The ability of IFN-γ to reproduce some of the cutaneous manifestations of delayed hypersensitivity was correlated with another series of experiments going on in our laboratory. Studies by Andrew Luster in collaboration with Jay Unkeless and Jeffrey Ravetch indicate that IFN-γ can induce macrophage and nonleukocytes to make a putative chemotactic factor (40). Shortly after the addition of IFN-γ (± 30 minutes) a gene termed γ-IP10 was induced. The complementary DNA was isolated and was shown to code for a small 10,000-mol wt polypeptide. A homology search through the National Biomedical Research Foundation Library indicated a significant relationship to platelet factor 4, a constitutent of the α-granule of human platelets. This gene could also be turned on when IFN-γ was added to human umbilical vein endothelial cells and fibroblasts. The function of platelet factor 4 is thought to be related to its

chemotactic activity, whereby it can direct the homing of neutrophils and monocytes under in vitro conditions (41). It will be of interest to evaluate the production of γ-IP10 in the environment of a delayed-type reaction utilizing specific probes for its message.

The capacity of IFN-γ to induce gene expression in macrophages and other "nonprofessional" immunocytes (keratinocytes, fibroblasts, endothelial cells) suggests a more global response in the milieu of delayed-type reactions. It is already clear that, in addition to macrophages, IFN-γ also induces the gene coding for the 10,000-mol wt polypeptide in human endothelial cells and fibroblasts. Such products and perhaps others coded for by other IFN-γ-induced genes (e.g., Ia) influence not only the homing and localization of T cells and macrophages but also the antimicrobial environment of nonleukocytes such as fibroblasts (42). This recruitment of the nonprofessional immunocyte adds a complexity to delayed reactions which was previously unsuspected.

Summary

Leprosy offers unusual opportunities for the study of cell-mediated immunity in humans. Manifestations of the disease result from the intracellular replication of *M. leprae* within dermal macrophages and Schwann cells leading to cutaneous lesions and peripheral neuropathy. In lepromatous leprosy, the selective inability of T helper cells to respond to *M. leprae* antigens and their absence from the dermal lesions serve as primary immunologic defects. The role of suppressor T cells, adherent monocyte

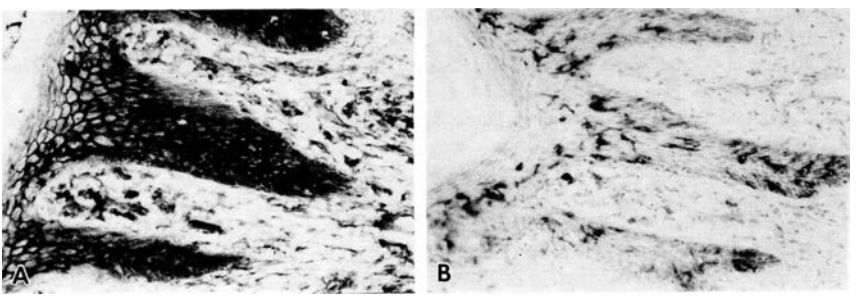

Figure 7. Keratinocytes and Langerhans cells in a cutaneous leishmaniasis lesion. (A) Photomicrograph of anti-Ia monoclonal staining of the epidermal cells of a cutaneous lesion. Extensive epidermal thickening and Ia staining of keratinocytes are observed; ×105. (B) Photomicrograph of OKT6 staining of Langerhans cells of the same cutaneous lesion. Staining of dendritic cells scattered between the keratinocytes is observed; ×105.

populations, accessory cells, and various *M. leprae* antigens on immune responsiveness is under consideration. We suspect that without the significant local production of lymphokines, e.g., interleukin 2 and IFN-γ, mononuclear phagocytes remain inactive and serve as permissive hosts for the organism. In tuberculoid disease, a more pronounced cell-mediated reaction is achieved and organism numbers are controlled. Recent evidence suggests that T cell products, generated in the local milieu of a delayed-type reaction, modify other cells such as epidermal keratinocytes, fibroblasts, and endothelial cells leading to their participation in cell-mediated immunity.

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Immunologic Regulation of Cutaneous Leishmaniasis

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Introduction

All leishmanial species are obligate intracellular protozoan parasites of the mononuclear phagocyte system, with selectivity towards a restricted range of vertebrate hosts and sandfly vectors. Animal reservoirs for clinical leishmaniasis include dogs, rodents, and arboreal mammals. The disease is transmitted by sandflies that ingest infected host macrophages. These contain amastigotes that transform into promastigotes in the sandfly gut. The extracellular flagellated promastigotes in turn can be injected into new vertebrate hosts as the insects feed. Both amastigotes and promatigotes can now be grown in large quantities in tissue culture.

There are at least three main categories of clinical leishmaniasis:

(a) Cutaneous leishmaniasis. This category is caused by *Leishmania major* or *Leishmania mexicana* species, and the disease evolves chronically and heals slowly. Less frequently, the disease can become nonhealing, diffuse disseminating, and relapsing (recidiva).

(b) Mucocutaneous leishmaniasis. This disease is due to *Leishmania braziliensis* species, and it involves delayed metastasis to the nasal and oropharyngeal regions with tissue destruction and gross mutilation (espundia).

(c) Visceral leishmaniasis. This is a systemic disease caused by *Leishmania donovani* and involves progressive spread of parasites through the endothelioreticular system. The disease, known as Kala-Azar, has a very high mortality rate within 2 years if untreated.

Understanding of immunologic control in leishmaniasis has been greatly facilitated by the use of inbred mouse strains that are susceptible to most *Leishmania* species pathogenic for humans. A wide spectrum of disease patterns can occur according to the genetic background of the host. So far, mapping of regulatory genes and analysis of mechanisms whereby their control is expressed have been studied predominantly with *L. donovani* and *L. major*. The classic studies of Bradley and his colleagues (1–3) established two levels of genetic expression affecting *L. donovani* infection in mice. First, innate susceptibility based on the relative resistance of the

macrophage during the early nonimmune phase and determined by the *Lsh* gene. Second, the efficacy of the immune stage for which three regulatory genes have been identified: the H-2 linked gene Rld-1 (3), an H-11 linked gene (4), and the Ir-2 gene (5). The *Lsh* gene has been mapped to a position on chromosome 1 between the Id-1 marker and the centromere (2) and is apparently identical to the *Ity* gene controlling the susceptibility to *Salmonella typhimurium* infection (6) and the *Bcg* gene determining the resistance to *Mycobacterium bovis* (7). The genetics of susceptibility to *L. major* are not yet as well defined. This infection is, nevertheless, very well suited for immunologic analysis, in that all grades of disease ranging from self-healing cutaneous lesion to uniformly fatal visceral leishmaniasis can be obtained with the same organism depending on the genetic constitution of the host. For example, strain A and CBA mice are highly resistant in that only minimal lesions develop after subcutaneous infection with 2×10^5 *L. major* promastigotes. B10.D2 mice are able to contain the disease whereas the lesions in DBA/1 mice develop slowly. In contrast, BALB/c mice are highly susceptible in that the disease is dose-independent and they succumb to uniformly fatal visceralizing disease even with minimal infecting doses. Genetic analysis in terms of separate innate and immune stages is less readily made with *L. major* than with *L. donovani*. It is likely that a very high level of innate susceptibility would overide the curative potential of any acquired immunity in mice such as BALB/c and SWR. The "BALB/c type" disease presentation was found to be controlled by a single major non-H-2-linked autosomal gene (8–10). Recent evidence has mapped this susceptibility to cutaneous leishmaniasis (*Scl*) gene provisionally to chromosome 8 (11), and hence clearly it is not identifiable with *Lsh*. One regulatory gene that exerts an effect common to both *L. donovani* and *L. major* is the alternative H-11^b allele in B10.129 (10M) congenic mice. The effect is equally pronounced in both systems (4). A weak H-2-linked influence has also been found on the outcome of *L. major* infection in congenic mice (8, 12).

In the past few years, the immunogenetic control of leishmaniasis has been intensively investigated and large amounts of information have now been accumulated. In this chapter, attempts will be made to summarize some of the findings in this field with special reference to *L. major* infection in the highly susceptible BALB/c mice.

Nature of Curative Immunity against Leishmaniasis

Although antileishmanial antibodies have been shown in vitro to lyse promastigotes with complement (13, 14), to promote phagocytosis (15),

and to induce surface patching and capping on promastigotes and amastigotes (16), there is little evidence of a corresponding in vivo role for antibody in determining the outcome of infection. Some monoclonal antibodies (17) or their Fab fragment (18) have been shown to reduce the infectivity of promastigotes when mixed in large quantity with the parasites before infection (the Winn test), but they had no effect on the disease development if injected separately from the promastigotes. In fact, the available evidence argues very strongly against a protective role for antibody in controlling leishmaniasis:

(a) The relatively late appearance of a low level of antibody during healing makes it unlikely that antibody would be effective against the wholly intracellular nature of the parasite.

(b) The outcome of the disease in different inbred mouse strains is not correlated to the titer or isotype of the antibody response (19).

(c) Mice genetically selected for low antibody response (Biozzi AB/L, selection I) are highly resistant to *L. major* infection and develop small self-healing lesions despite the low level of antibody produced (20).

(d) C3H mice rendered wholly antibody-deficient by treatment from birth with sheep anti-μ chain mouse immunoglobulin (μ-suppression) are able to control and heal from an *L. major* infection indistinguishable from untreated mice (21).

(e) Prolonged administration of large amounts of hyperimmune serum or antibody fractions from donors protectively immunized against *L. major* fails to influence the incidence or outcome of infection in highly susceptible BALB/c mice (22).

(f) Prior exposure to certain specific monoclonal antibodies against *L. major* increases the incidence and severity of infection (23).

Collectively, these results are clearly incompatible with an important protective role for antibody.

In contrast, the case for a causal role of cell-mediated immunity (CMI) in acquired resistance to leishmaniasis is impressive:

(a) Resistant strains of mice rendered relatively T cell-deficient by thymectomy followed by irradiation and reconstitution with syngeneic bone marrow cells are less able to control *L. major* infection and have delayed healing (24).

(b) Athymic mutants of the highly resistant CBA and C57BL/6 mice are totally unable to control *L. major* infection which progresses and visceralizes (25). The normal resistance, however, can be completely restored by reconstituting the T cell-deficient nu/nu mutants with as few as 10^6 syngeneic T cells. The T cells involved have been identified as bearing the Lyt-1^+2^- cell surface marker.

(c) Acquired protective immunity against *L. major* and *L. donovani* in resistant CBA mice can be adoptively transferred by T cells but not by B cells (26, 27). In the case of *L. major*, the protective cells are again found to be Lyt-1$^+$2$^-$ T cells (28).

Because of the predominant intracellular location of the infecting parasites, it is reasonable to assume that the major leishmanicidal effector mechanism in vivo is the macrophage, which is activated by lymphokines produced by specific T cells. Intracellular killing of leishmania in vitro by macrophage activation with various lymphokine preparations has been reported by several laboratories (29–33). This involves either the generation of unstable oxygen metabolites or a nonoxidative killing mechanism (33). *L. major*-specific Lyt-1$^+$2$^-$ T-cell clones have been isolated that possess helper, delayed-type hypersensitivity (DTH), and macrophage-stimulating activities (34).

The role of cytotoxic T (Tc) cells in acquired immunity against leishmanial infection has also been considered (35). However, no convincing evidence for this mechanism has so far emerged (36). This is perhaps not surprising because the efficacy of Tc cells against infectious agents is based primarily on the destruction of host cells containing replicating pathogens which, when released prematurely, fail to reinfect other host cells. This generally works well for viruses but leishmania, which is highly infectious in the amastigote stage, could remain unaffected. Natural killer cells have been shown to play a role in the immunity against systemic *L. donovani* infections in C57BL/6 bg/bg (beige) mice but not in the resistance against cutaneous *L. major* infection (37).

Specific Suppression of Curative Immunity

The development in the highly susceptible BALB/c mice of fatal disseminating leishmanial infection is accompanied by a parallel induction of specific suppression of CMI. This includes *(a) Leishmania*-specific suppression of DTH (38), *(b)* antigen-specific impairment of macrophage-activating lymphokine generation (39), and *(c)* markedly reduced interleukin 2 (IL-2) production by the splenic T cells (40). The leishmania-specific antibody response, however, remains unperturbed.

In spite of this anergy, available evidence argues against any intrinsic defect in the ability of macrophages from BALB/c mice either to kill the parasite or to present antigen on their surface in an effective immunogenic form to activate T cells. First, there is no impairment in the initial induction or expression of DTH for the first 3 weeks after infection (41). Second, BALB/c mice that recovered from *L. major* infection after sublethal

dose irradiation expressed strong DTH-specific-reactivity and are refractory to subsequent *L. major* infection (38).

The active suppression of CMI has been associated with the generation of a potent population of specific suppressor T (Ts) cells (28, 38). This is based on the following observations:

(a) T cells from infected DTH-negative mice can adoptively inhibit both the induction and expression of *Leishmania*-specific DTH, without affecting responses to unrelated antigens (40).

(b) BALB/c mice exposed to sublethal (550 rads) gamma-irradiation prior to infection are able to control the disease and retain DTH reactivity. This outcome, however, can be entirely reversed and normal disease progression restored, if the irradiated animals are injected with as few as 10^6 T-cells isolated from the suppressed donors with progressive disease. Normal T cells can also effect this reversal, after a transient period of disease arrest, whereas T cells from cured donors transfer protective immunity (Figure 1) (38). Similar results were obtained with *L. donovani* infection in genetically susceptible B10.D2 mice (42).

(c) The disease-promoting T cells bear the Lyt-1^+2^- phenotype (25, 28). They are anologous with Ts cells previously found to suppress DTH to influenza virus and sheep erythrocytes, which are distinct from the Lyt-1^-2^+ Ts cells regulating antibody response (43).

(d) A cloned T cell line has been derived from a BALB/c mouse with progressive *L. major* infection (44). These Lyt-1^+2^- cells and their culture supernatants could produce specific suppression of T lymphocyte proliferation in vitro and the induction of DTH to *L. major* in vivo. When injected together with the infecting promastigotes, the cells significantly enhance *L. major* lesion development in BALB/c mice.

(e) BALB/c mice rendered wholely antibody deficient by anti-μ treatment from birth can also control *L. major* infection (21). This effect could be completely reversed by T cells from BALB/c mice with progressive lesions without the corresponding restoration of the antibody response.

Collectively, these findings argue forcibly for the contention that the genetically determined failure of a curative immune response against leishmaniasis is due to a central defect of the initial parasite handling ability of the host macrophages. Much greater proliferation of amastigotes occurs both within the cutaneous lesion (45) and in subsequent visceralization (46). The result would be the preferential generation of a potent population of Ts cells which inhibit the induction of curative CMI.

Ts cells in the present system are defined in purely operational terms in that they down-regulate protective immunity.

Several outstanding questions, however, remain unanswered:

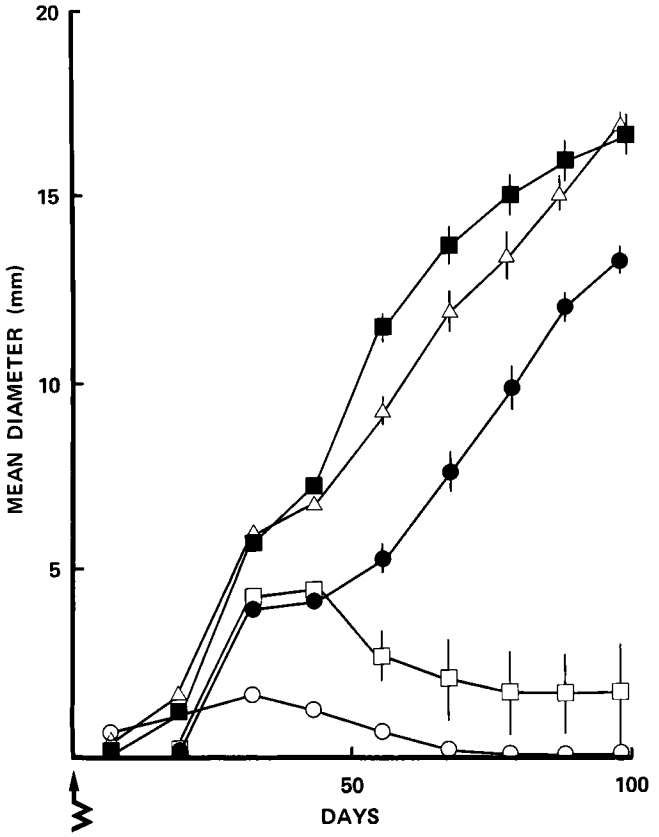

Figure 1. Prophylactic effect of 550-rad irradiation 24 hours before infection with 2 × 10^7 *L. major* promastigotes in BALB/c mice (□) compared with normal mice (■). This effect is abrogated by injection with 2–5 × 10^7 T cells from normal mice (●) or donors with progressive *L. major* infection (△). T cells from cured donors transfer immunity (○). From Howard et al. (38).

(a) What are the factors determining the preferential induction of Ts cells or T effector cells? In reconstitution experiments involving BALB/c nu/nu mice, 10^6 normal T cells were found to protect the nude mice, whereas 10^8 such T cells had the opposite effect (25). It is evident that the protective T cells and the counterprotective Ts cells may have different thresholds of activation.

(b) What is the mechanism of interaction between the Ts cells and the effector cells? Whether or not Ts cells exert their effect by release of a suppressor factor is at present conjectural. The possibilities remain that the Ts cells may affect the effector cells by a direct cytolytic action (47)

or conversely by indirect competition with the effector cells for growth factors or target macrophages.

(c) What are the cellular characteristics of Ts cells and effector T-cells? So far the distinction between the two subsets of T-cells is purely a functional one. They both express the Lyt-1^+2^- cell surface marker. However, it has recently been demonstrated that antigen-specific, Lyt-1^+2^- L3T4^+ T cells, which mediate DTH in vivo and activate macrophage leishmanicidal activity in vitro, can nevertheless exacerbate *L. major* disease progression when injected into normal recipients (48, 49). Furthermore, it has been observed that T cells bearing this phenotype predominate in the spleen and lymph nodes of infected BALB/c mice as compared to resistant CBA mice (50). It has therefore been argued that the fatal progressive disease in the BALB/c mice is due to the overstimulation of CMI and that the functional expression of suppression or protection merely reflects the quantitative states of the same cell population. While this remains a theoretical possibility, the question of how such cells are preferentially activated in susceptible, compared to resistant, strains is still unclear.

Resistance to Macrophage Leishmanicidal Activity

In striking resemblance to the clinical espundia due to *L. braziliensis*, C57BL/6 mice infected with *L. mexicana amazonensis* develop small but persistent lesions which eventually increase locally and lead to destructive metastases in the nasal region (51). The chronicity and eventual progression of the disease apparently occur in the presence of sustained CMI as measured by the expression of strong DTH throughout the infection. The implication of this clinical and experimental observation is that, despite continued exposure to lymphokine activation via a persistent CMI response, the host macrophages fail to eliminate this strain of parasite. Experimental evidence for this has now been obtained (52). *L. mexicana amazonensis* was found to resist killing within lymphokine-activated C57BL/6 macrophages whereas *L. major* and *Toxoplasma gondii* were wholly susceptible in parallel experiments. The resistance appears to be intrinsic to the *L. mexicana amazonensis* and not due to failure of macrophages to respond to lymphokine activation because the cells are able to kill *L. major* and *T. gondii* in double infections with the resistant protozoa. An important question that arises from these observations is whether such refractoriness to the normally curative CMI would be an obstacle to prophylactic immunization with the relevant species. The mechanism of evasion and

the extent to which it is operative in leishmaniasis therefore merits further investigation.

Experimental Prophylactic Immunization

To date, the only successful immunization strategy against leishmaniasis has been limited to Old World cutaneous leishmaniasis using small doses of living virulent *L. major* at a selected site. This high-risk procedure, though justifiable in highly endemic areas in the absence of other effective methods, is obviously not acceptable for mass vaccination. The effectiveness of immunization with killed vaccine against clinical leishmaniasis is at present unclear. In experimental animal models a weak protection could be induced against self-curing *L. major* infection in genetically resistant CBA and C3H mice with ultrasonicated promastigotes (26). Attempts to protect against more severe diseases such as those caused by *L. donovani* or *L. major* in BALB/c mice with variously killed promastigotes have only met with partial success (53–55). However, recent studies with lethally irradiated, heat-killed (22, 56) or sonicated promastigotes (57) of *L. major* have been much more favorable in outcome. The characteristics of prophylactic immunization against this otherwise fatal infection will be discussed in this and the next section.

BALB/c mice given repeated intravenous (i.v.) injections of lethally irradiated (150,000 rads) or heat-killed promastigotes develop substantial protection against *L. major* challenge infection. Immunity is expressed kinetically by the delayed appearance of the cutaneous lesion which progresses slowly, whereas complete protection can be achieved with smaller doses of challenge ($<10^6$ promastigotes). For optimal effect the i.v. route is superior to the intraperitoneal (i.p.) in protecting against higher challenge doses, whereas subcutaneous (s.c.) or intramuscular (i.m.) injections are totally ineffective with or without adjuvants. Large immunizing doses of 10^7 are required and repeated administration is generally more potent than single. The protection induced is long-lasting (>150 days) and effective against both promastigote and amastigote stage challenge. The immunity against *L. major* infection is also inducible with irradiated *L. donovani* promastigotes while protection induced by *L. major* extends to other cutaneous leishmaniases—*L. mexicana, L. amazonensis* and *L. panamensis* (56).

The immunologic features of prophylactically immunized BALB/c mice are strikingly different from those characteristic of convalescent immunity (58). Specific antibody responses are substantially higher than those found in mice either cured of infection or with progressive disease. How-

ever, impairment of the humoral response by prior splenectomy does not influence the induction of protective immunity, indicating a lack of causal relationship between antibody and protective immunity. This is supported by the failure to confer protection by repeated and prolonged passive transfer of large amounts of hyperimmune serum or its isotype fractions (22). Protective immunity is, however, passively transferable with T cells and not B cells derived from the spleen and lymph nodes of immunized donors. The protective T cells again express Lyt-1^+2^- cell surface phenotype (58). Prophylactic i.v. immunization does not induce any detectable cytotoxic T cell response or DTH. This latter feature is in sharp contrast to that seen in mice recovered from infection that express strong DTH to all *L. major* antigen preparations tested (58). Spleen, lymph node, and peritoneal exudate cells from protectively immunized donors also fail to transfer DTH locally or systemically. Even more unexpectedly, the protective T cell population strongly and specifically suppresses DTH induced by killed promastigotes (59). These results imply that the protective T cells arising from prophylactic immunization are likely to be distinct from those determining convalescent immunity.

Impairment of Prophylactic Immunization

As mentioned earlier, the parenteral routes (i.v. and i.p.) are mandatory in the induction of protective immunity. Further studies have revealed that BALB/c mice injected with irradiated promastigotes via the s.c. route show disease earlier after infection and accelerated progression compared with uninjected controls (49). Similar disease exacerbation has also been found after s.c. immunization with freeze-thawed promastigote antigens in Freund's complete adjuvant (50). Even more strikingly, s.c. immunized mice were found to be highly refractory to subsequent prophylactic immunization (60). The inhibitory effect can be achieved with a single s.c. or i.m. injection although less potently than with four and is even effective against four repeated i.v. immunizations. Once induced, the effect persists undiminished for at least 100 days. A weaker effect is also induced by s.c. injections given after i.v. immunization (Figure 2). As for i.v. protective immunization, the blocking effect of s.c. injection is not dependent on continuing viability of the promastigotes, being induced equally readily with heat-killed, formalin-fixed, or sonicated parasites. The phenomenon is specific for leishmanial antigens, extends to mouse strains genetically resistant as well as susceptible to *L. major* and, in congenic mice of BALB background, is independent of the major histocompatibility (H-2) gene complex.

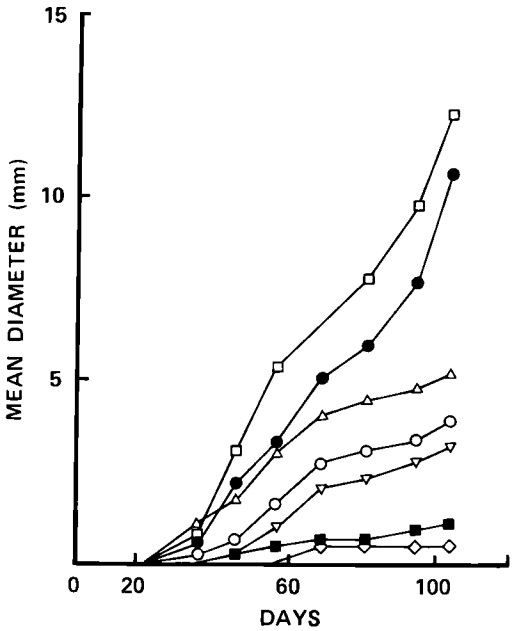

Figure 2. Effect of s.c. injection of irradiated *L. major* promastigotes after intravenous immunization on the subsequent disease developments. Groups of 5 BALB/c mice were given one or four times weekly i.v. immunizations followed by one or four times weekly s.c. injections with irradiated promastigotes. Together with uninjected controls (●), they were infected with 2 × 10^5 *L. major* promastigotes and the subsequent lesion development followed. (□) one time s.c. injection alone; (▽), one time i.v. immunization alone; (■), four times i.v. immunization alone; (△), one time i.v. + four times s.c.; (□) four times i.v. + four times s.c.; (◇) four times i.v. + one time s.c.

Multiple s.c. injections of irradiated *L. major* promastigotes do not induce significant levels of specific serum antibody nor do they inhibit subsequent antibody response of any major isotype to i.v. immunization. Instead, some priming effect of s.c. injection is detectable. The same s.c. injections also induce specific DTH, which can be transferred locally or systemically. Despite this evidence of a DTH/helper type of T cell response, transfer of T cell-enriched spleen cells from multiple s.c. immunized donors to normal recipients completely abrogate the protective response to i.v. immunization. Conversely, T cell-depleted spleen cells are without effect. The inhibitory T cells again express the Lyt-1^+2^- L3T4^+ phenotype. In cotransfer experiments, the T cells from s.c. immunized donors counteract completely the protective effect of T cells from i.v. immunized donors in 550 rad-irradiated recipients. They are as potent as T cells from donors

with progressive infection both in this capacity and in abrogating the prophylactic effect of sublethal irradiation itself (60).

Discussion and Conclusion

It appears that four functionally separate subpopulations of T cells can be induced during infection or immunization with *L. major* in BALB/c mice (Table I). Two of these are protective (Tr, T cells from mice recovered from infection, and Ti, T cells from i.v. immunized mice), whereas the other two are counterprotective (Ts, suppressor T cells from mice with progressive lesions, and Tsc, T cells from mice after s.c. injections). They are thus far indistinguishable according to cell surface phenotype. Tr cells, which mediate strong DTH, help antibody synthesis, and produce IL-2 in response to suboptimal concentrations of concanavalin A (E. Cillari and F. Y. Liew, unpublished), are protective against *L. major* infection. Ti cells, which are also protective, produce high levels of IL-2 but suppress specific DTH reactivity. In contrast, Tsc cells, which mediate DTH, help antibody synthesis, and produce IL-2, are strongly counterprotective. Ts cells, however, not only exacerbate lesion development, but suppress the induction of DTH and the production of IL-2 by Tr, Ti, and normal T cells (Cillari and Liew, unpublished).

It could be argued that, although the protective/curative mechanism involves macrophage activation by specifically sensitized T cells, cuta-

Table I. Four Functionally Distinct Subpopulations of T Cells Induced during Infection or Immunization with Leishmania major

Cells	*Source of cells*	*DTH*	*Help in antibody synthesis*	*IL-2*	*Protection*	*Suppression*
Tr	Cured infection	+ +[a]	+	+	+	–
Ti	i.v. immunization	–	+ +	+	+	–
Ts	Progressive infection	–	+	–	–	+
Tsc	s.c. immunization	+	±	+	–	+

Tr, T cells from mice recovered from infection; Ti, T cells from i.v. immunized mice; Ts, suppressor T cells from mice with progressive disease; Tsc, T cells from mice injected s.c. with killed parasites. From Liew and Howard (61).

a. +, strongly positive; ±, modest but significant priming; –, negative.

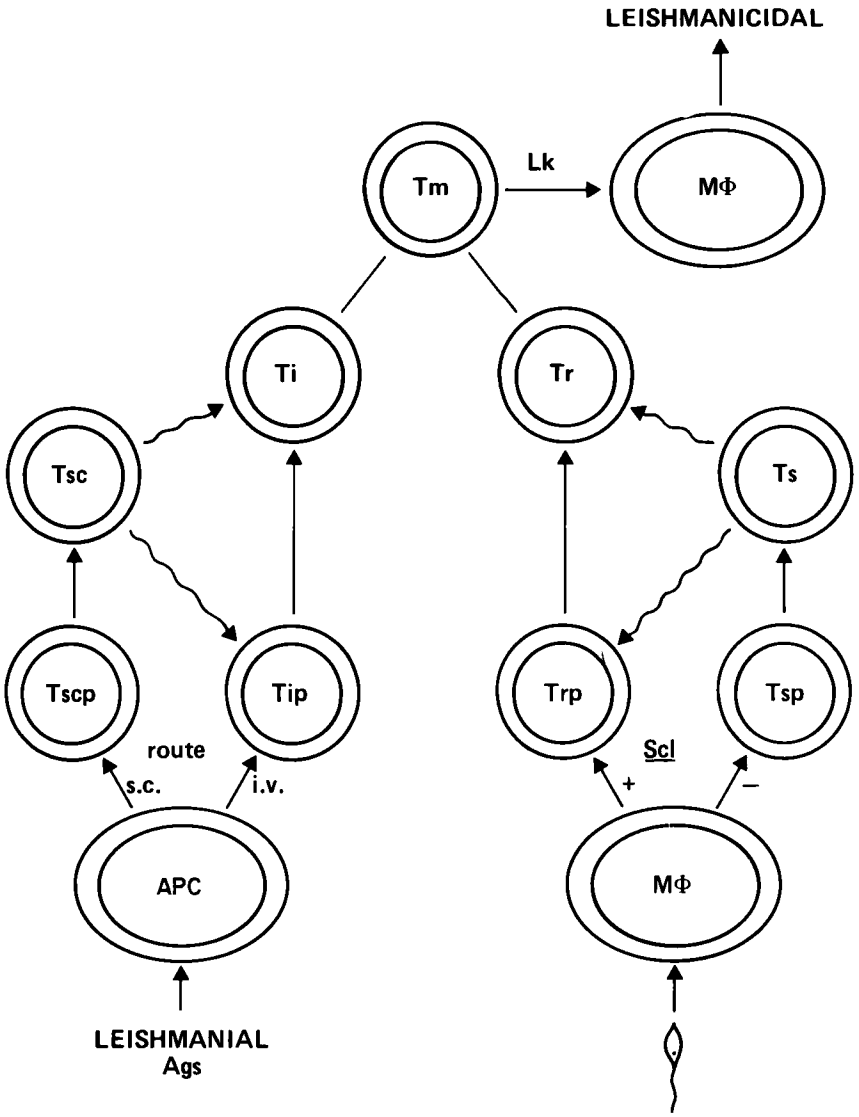

Figure 3. Schematic representation of the regulation of protective immunity against cutaneous leishmaniasis by infectious or killed *L. major.* Mϕ, macrophages; Lk, lymphokines; Tm, macrophage-activating T cells; Ti, T cells from i.v. immunized mice; Tip, precursor of Ti; Tr, T cells from mice recovered from infection; Trp, precursor of Tr; Tsc, T cells from mice injected s.c. with killed parasites; Tscp, precursor of Tsc; Ts, suppressor T cells from mice with progressive disease; Tsp, precursor of Ts; APC, antigen-presenting cells; *Scl*, gene controlling susceptibility to cutaneous leishmaniasis. Undulatory arrows denote suppression.

neous DTH is a dissociable accompanying manifestation that is neither mandatory nor variably present. The lymphokines responsible for the manifestation of DTH could be distinct from those mediating protective CMI. Thus, Ti cells from mice immunized four times may be identical to the T cells in convalescent animals, with both cell types possessing the capability to confer protective immunity against infection. Similarly, Tsc cells could be either exactly the same as Ts cells or inducers of Ts cells with the ultimate in vivo expression of suppression of protective effector T cells.

An alternative interpretation would be that the host-protective effector T cells are heterogenous. Protective T cells induced by i.v. immunization with killed promastigotes could be different from those determining convalescent immunity subsequent to sublethal irradiation before infection. Thus, dual protective mechanisms against cutaneous leishmaniasis may exist, each with its distinct stimulatory T cells (Ti or Tr cells in Figure 3). Whether or not these represent distinct cell lineages or merely differentiation stages of the same line is unknown. Each of these effector cells has its own complementary regulatory mechanism. Tr cells are suppressed by Ts cells, the induction of which depends on *Scl* gene expression in the macrophages, whereas Ti cells are controlled by Tsc cells, which are induced when the antigens are presented via percutaneous antigen presenting cells. The similarity observed between the ability of Tsc cells and Ts cells to reverse the combined protective potential of transferred Ti cells and sublethal irradiation could represent a similar end result of dissimilar cellular interactions. This interpretation implies that, although DTH measures one facet of macrophage activation by lymphokines, its functional expression is not obligatory for effective intracellular parasiticidal activity.

The inhibition of prophylactic immunization as a result of s.c. injection of *L. major* antigens is not a trivial observation. Although a parallel effect has so far not been reported for other parasitic infections, it may nevertheless have important general practical implications. If this phenomenon in the murine model extends to clnical leishmaniasis and to isolated molecularly defined antigens, then future strategy for vaccination against this disease will have to circumvent a formidable obstacle.

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Macrophage Activation in the Acquired Immunodeficiency Syndrome

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If one draws up a list of the microbial pathogens against which successful host defense is thought to require an intact cell-mediated immune response (1) and compares this to a list of the pathogens that regularly infect patients with the acquired immunodeficiency syndrome (AIDS) (2) (Table I), the two lists are virtually superimposable. In addition, many of the AIDS pathogens are known to be intracellular organisms that, although capable of causing quite different clinical manifestations, share the common feature of being able to enter and replicate within unstimulated macrophages (1). In the presence of a normal, effective T cell-dependent immune response, however, which is presumed to be largely mediated by antigen-triggered lymphokine secretion with resultant macrophage activation, these same intracellular pathogens are killed or their growth is inhibited. Thus, the particular spectrum of opportunistic microorganisms that consistently infect AIDS patients, the frequent failure of these patients to respond to appropriate antimicrobial therapy, their propensity for relapse despite adequate treatment, and their eventual uniformly fatal outcome all serve to indicate graphically that the process of macrophage activation in patients with AIDS is either profoundly disordered or, more likely, has not taken place at all (3). Histopathologically, these clinical observations are expressed by the absence of granuloma formation, little or no inflammatory cell infiltrates, and overwhelming numbers of microorganisms in widespread and numerous foci of tissue infection.

Given the large amount of currently available information that has clearly documented that T cell function in AIDS is strikingly impaired (4), the remarkable susceptibility of AIDS patients to intracellular pathogens is not only *not* surprising but is predictable. With the concept of T cell-dependent macrophage activation in mind, the studies reviewed in this chapter have been directed at several interrelated questions concerning the immunopathogenesis of opportunistic infections in AIDS: (*a*) Are AIDS patients' T cells capable of generating macrophage-activating lymphokines? (*b*) Are AIDS patients' mononuclear phagocytes intrinsically defective in antimicrobial activity? and (*c*) Can AIDS patients' mac-

Table I. Opportunistic Pathogens in AIDS

Pneumocystis carinii
Toxoplasma gondii
Cryptosporidium
Isospora belli
Cytomegalovirus
Herpes simplex virus
Candida albicans
Histoplasma capsulatum
Cryptococcus neoformans
Salmonella sp.
Mycobacterium avium-intracellulare

See Reference 2 for additional detail.

rophages express the activated state once provided with exogenous activating lymphokines?

Lymphokine-generating Capacity of AIDS T Cells

Crude Lymphokines

In response to stimulation with either nonspecific mitogens or specific microbial antigens, peripheral blood mononuclear cells (PBMC) from healthy individuals readily secrete soluble T cell products (lymphokines), which in vitro activate both human monocyte-derived and tissue-derived (alveolar) macrophages as judged by two linked expressions of the activated state—augmented oxidative metabolism (e.g., H_2O_2 release) and enhanced antimicrobial activity against intracellular pathogens (5–9). In the absence of lymphokine stimulation, these macrophages are normally quiescent and generate little H_2O_2 and support microbial replication. In our initial study of AIDS patients with opportunistic infections, we tested AIDS PBMC for the ability to generate these crude macrophage-activating lymphokines (3). We found several patients whose cells could

Table II. Failure of AIDS Peripheral Blood Mononuclear Cells to Generate Crude Macrophage-activating Lymphokines

Normal macrophages stimulated with[a]	*Antimicrobial activity*		*Oxidative activity (fold increase in H_2O_2 release)*
	% toxoplasmas killed	*% inhibition of C. psittaci replication*	
Medium alone	4 ± 2	0	—[b]
Con A-lymphokines from			
Controls	38 ± 5	84 ± 3	5.9 ± 1.4
AIDS patients	9 ± 4	21 ± 7	1.5 ± 0.3
Antigen-lymphokines from			
Controls	52 ± 3	85 ± 2	5.0 ± 0.7
AIDS patients	2 ± 1	5 ± 3	1.0 ± 0.2

a. Monocyte-derived macrophages from healthy controls were treated for 72 hours before infection or assay with medium alone or 10% mitogen-induced or antigen-induced lymphokines generated from cultures of mononuclear cells from controls or patients with AIDS. Lymphokines induced by antigen (toxoplasma antigen) were from subjects with positive serum Sabin-Feldman dye tests (3).

b. Control macrophages treated with medium alone released 104 ± 33 nmol of hydrogen peroxide (H_2O_2) per milligram of protein in 90 minutes.

Table III. Production of Interferon Gamma by Mononuclear Cells from AIDS Patients with Opportunistic Infections

	IFN-γ production		
Subjects	*Con A*	*T. gondii antigen*	*C. albicans antigen*
Healthy controls			
Range	300–6,000	300–3,000	100–300
Mean ± SEM	1,884 ± 194	1,108 ± 93	200 ± 41
AIDS patients (no.)			
1	40	—[a]	<10
2	20	—	—[a]
3	350	—	<10
4	150	<10	—
5	400	<10	—
6	20	<10	—
7	60	<10	<10
8	120	<10	—
9	1,000	20	<10
10	<10	—	<10
11	300	<10	—
12	20	<10	—
13	<10	<10	—
14	10	<10	—
15	10	<10	—
16	2,000	—	—

a. Not applicable because either the serum Sabin-Feldman dye test was negative or there was no evidence of mucosal candidiasis (3).

produce active lymphokines in response to mitogen (concanavalin A); no patient, however, responded to specific antigen with the production of effective activating lymphokines (Table II) (3). This T cell deficit also correlated closely with lymphocyte proliferative activity; responses to mitogen were highly variable (absent to near-normal), whereas antigen-induced proliferation was typically absent (3).

Interferon Gamma

Because interferon gamma (IFN-γ) has been recently shown to be a key macrophage-activating component of crude T cell-derived lymphokines (5–7, 9–13), we also examined the capacity of PBMC from AIDS patients with opportunistic infections to generate specifically this T cell product. As shown in Table III, cells from one-third of the patients studied secreted normal IFN-γ levels (≥300 U/ml) in response to mitogen (concanavalin A). In response to previously encountered and clinically relevant microbial antigens, however, 13 of 14 tested patients failed to secrete any detectable IFN-γ (<10 U/ml); the one responder produced a barely measurable level (20 U/ml) (3). We have now extended this analysis to a total of 28 AIDS patients with opportunistic infections, and have found that the failure to produce any antigen-stimulated IFN-γ is typical of these patients (Table IV), and can be considered the functional immunophenotype of AIDS patients whose immune deficit has progressed sufficiently to render them subject to opportunistic infections. Because one-third of these patients secreted normal levels of IFN-γ in response to nonspecific mitogen stimulation, it is clearly preferable and we believe more accurate to test T cell function in patients with AIDS or the AIDS prodrome (AIDS-related complex [ARC]) using only specific antigens. In our laboratory, we rou-

Table IV. Interferon Gamma Production by Twenty-eight AIDS Patients with Opportunistic Infections

	IFN-γ production		
T cell stimulus	*Normal*[a]	*Abnormal but detectable*	*Undetectable*
Concanavalin A	32%	48%	20%
Specific antigen[b]	0%	19%	81%

a. Normal IFN-γ production in response to stimuli used: concanavalin A, 1,401 ± 160 U/ml (range 300–10,000); *T. gondii* antigen, 986 ± 149 U/ml (range 200–3,000); cytomegalovirus antigen, 773 ± 140 U/ml (100–3,800); and *C. albicans*, 200 ± 41 U/ml (100–300).

b. Previous exposure documented by serologic testing or clinical findings. Antigens included *T. gondii*, cytomegalovirus, and *C. albicans*.

Table V. Interleukin 2 Production by AIDS Mononuclear Cells in Response to Specific Microbial Antigen[a]

Patients	IFN-γ production		
	Normal[b]	*Abnormal but detectable*	*Undetectable*[c]
Total group ($n = 32$)	1/32 (3%)	10/32 (31%)	21/32 (66%)
Opportunistic infections ($n = 21$)	0/21 (0%)	6/21 (29%)	15/21 (71%)
Kaposi's sarcoma ($n = 11$)	1/11 (9%)	4/11 (36%)	6/11 (55%)

a. Antigens included *T. gondii*, tetanus toxoid, cytomegalovirus, *C. albicans*, and *Herpes simplex* virus (14).

b. Normal IL-2 production in response to these antigens (14): *T. gondii*, 33.9 ± 2.7 U/ml (14–45); tetanus toxoid, 11.9 ± 1.8 U/ml (2.2–20), cytomegalovirus, 3.6 ± 0.5 U/ml (2.0–4.8); *C. albicans*, 20.2 ± 4.2 U/ml (5.3–40); and *Herpes simplex* virus, 9.9 ± 1.7 U/ml (2.8–19).

c. <0.1 U/ml of IL-2 (14).

tinely utilize (*a*) *Toxoplasma gondii* lysate antigen for patients with positive serum Sabin–Feldman dye tests, (*b*) *Candida albicans* sonicate antigen for those with oral candidiasis or the few patients with positive *Candida* skin tests, (*c*) cytomegalovirus (CMV) antigen if the serum complement fixation titer is ≥1:8, or (*d*) tetanus toxoid for those previously immunized (3, 14).

It is also worth pointing out that although most of our AIDS patients have been quite lymphopenic with markedly decreased numbers of peripheral blood $T4^+$ cells and that this quantitative abnormality alone may largely explain the failure to proliferate or produce IFN-γ in response to soluble antigen, we have observed several patients with full-blown AIDS (opportunistic infections) who have normal absolute numbers of circulating $T4^+$ cells. Nevertheless, these patients' PBMC also failed to secrete IFN-γ in response to specific antigen. We presume this observation reflects cytopathic (but noncytolytic) effects induced by human T lymphotrophic virus III (HTLV-III) infection.

Interleukin 2

Because interleukin 2 (IL-2), another T cell lymphokine implicated in a variety of cellular immune reactions, is readily produced by normal

cells in response to mitogen and antigen (14), we also measured the IL-2 activity in the supernatants of the AIDS patients' PBMC cultures. Although IL-2 by itself does not directly activate macrophages (10), our interest in IL-2 generation stemmed from prior observations indicating that the presence of IL-2 plays an important role in the capacity of T cells to produce IFN-γ (15). As shown in Table V, PBMC from only 1 of 32 (3%) AIDS patients with opportunistic infections or Kaposi's sarcoma secreted normal levels of IL-2 in response to a specific antigen, and 21 (66%) failed to produce any detectable IL-2 (14). Thus, deficient antigen-induced IL-2 production also appears to be typical of patients with or at high risk for developing opportunistic infections. If IL-2 is indeed required for T cell production of IFN-γ (14, 15), these results suggest that impaired IFN-γ secretion may largely reflect or in fact be due to absent antigen-stimulated IL-2 generation.

AIDS Monocyte and Macrophage Function

Monocytes

The second question we asked was whether AIDS mononuclear phagocytes might also be defective in antimicrobial effector cell function. In these studies, we tested AIDS monocytes for their ability to (*a*) stain for myeloperoxidase, (*b*) generate and release H_2O_2 (a key antimicrobial oxygen intermediate), (*c*) respond to both inert (zymosan) and viable (*Toxoplasma gondii*) phagocytized targets with oxidative burst activity, and (*d*) to kill or inhibit *T. gondii* and *Chlamydia psittaci*. These latter two intracellular pathogens were selected for use in order to test the effectiveness of both the oxygen-dependent and oxygen-indpendent mechanisms of the AIDS monocyte. Activity against *T. gondii* is primarily oxygen-dependent (11), whereas oxygen-independent mechanisms are sufficient to inhibit *C. psittaci* replication (16). As shown in Table VI, monocytes from AIDS patients with opportunistic infections were indistinguishable from monocytes from healthy controls with regard to both oxidative and in vitro antimicrobial activities (3), indicating that AIDS peripheral blood mononuclear phagocytes showed neither intrinsic nor acquired defects in intracellular effector function.

Macrophages

Given that AIDS monocytes appeared to behave in a normal fashion, we proceeded to test AIDS macrophages for the capacity to respond to lymphokines, and once stimulated, to express the activated state by displaying enhanced antimicrobial activity. In these experiments, AIDS

Table VI. Activities of AIDS Peripheral Blood Monocytes and Monocyte-derived Macrophages

	Oxidative activity		*Antmicrobial activity*	
	H_2O_2 release nmol/mg/90 min	*Fold increase in H_2O_2 release*	*% toxoplasmas killed at 4 hours*	*% inhibition of C. psittaci at 20 hours*
Monocytes[a]				
Control	939 ± 61	—	38 ± 7	86 ± 3
AIDS	877 ± 55	—	40 ± 5	81 ± 3
Macrophages stimulated with[b]				
Medium alone				
Control	115 ± 26	—	2 ± 1	0
Normal lymphokines				
Control	—	4.6 ± 0.6	43 ± 4	88 ± 3
AIDS	—	5.6 ± 0.4	39 ± 3	89 ± 3
IFN-γ				
Control	—	5.8 ± 0.5	41 ± 3	89 ± 4
AIDS	—	6.2 ± 0.7	37 ± 4	90 ± 3

See Reference 3 for additional details.

a. Monocytes were cultivated 1 day before infection or assay.

b. Monocytes were first cultivated for >7 days, then treated for 72 hours with medium alone, 10% active (normal) lymphokines, or 300 U of partially purified or recombinant IFN-γ per milliliter.

monocyte-derived macrophages were cultivated with crude lymphokines prepared from cells from healthy donors. As shown in Table VI, once properly stimulated, macrophages from AIDS patients with opportunistic infections readily generated increased levels of H_2O_2 and exerted normal levels of enhanced intracellular activity against both *T. gondii* and *C. psittaci* (3). As in our experiments with AIDS monocytes, these in vitro observations also suggested that AIDS macrophages had no defect in either the ability to respond to exogenous lymphokines with activation or once activated to exert effective antimicrobial activity.

To extend this analysis from the peripheral blood-derived macrophage to the tissue macrophage, we also studied alveolar macrophages from 11 AIDS patients undergoing diagnostic bronchoalveolar lavage (9). After 72 hours in culture, unstimulated alveolar macrophages from all individuals examined (3 healthy volunteers, 12 non-AIDS patients, 11 AIDS patients) released comparably low levels of H_2O_2 and readily supported the intracellular replication of both *T. gondii* and *C. psittaci* (Table VII). Cells cultivated with crude (normal) lymphokines during this 72-hour period, however, displayed the activated phenotype in that they produced two- to threefold more H_2O_2 and exerted both antitoxoplasma and antichlamydial activity (Table VII and Figure 1). Once similarly activated, AIDS alveolar macrophages behaved identically to cells from the controls (9). It is also worth pointing out that AIDS alveolar macrophages were fully capable of being activated despite being obtained from an in vivo environment in which both pulmonary Kaposi's sarcoma and opportunistic infections had developed (9).

AIDS Macrophage Response to Interferon Gamma

Because treatment with IFN-γ is one of the immunotherapeutic avenues now being tried in AIDS patients, we also utilized the preceding models to determine the in vitro responsiveness of AIDS macrophages to this particularly key activating lymphokine (5–7, 9–13). As shown in Tables VI and VII, AIDS peripheral blood- and tissue-derived (alveolar) macrophages readily responded to treatment with partially purified and recombinant IFN-γ, and attained levels of activation identical to that displayed by in vitro activated macrophages from healthy controls (3, 9).

Conclusion

From the preceding in vitro studies, it seems reasonable to conclude that both the peripheral blood monocyte and the tissue macrophage in AIDS

Table VII. Responsiveness of AIDS Tissue (Alveolar) Macrophages to Activating Lymphokines and Recombinant Interferon Gamma

Source and treatment of alveolar macrophages	*Oxidative activity (fold increase in H_2O_2 release)*	*Antimicrobial activity*	
		Fold increase in Toxoplasmas/100 Cells	*% inhibition of C. psittaci replication*
Healthy controls			
Medium alone	—	7.7	0
Lymphokine	2.5	2.7	74
IFN-γ	3.1	2.4	67
AIDS patients			
Medium alone	—	7.4	0
Lymphokines	2.2	2.4	71
IFN-γ	2.5	1.8	78

See Reference 9 for additional detail.

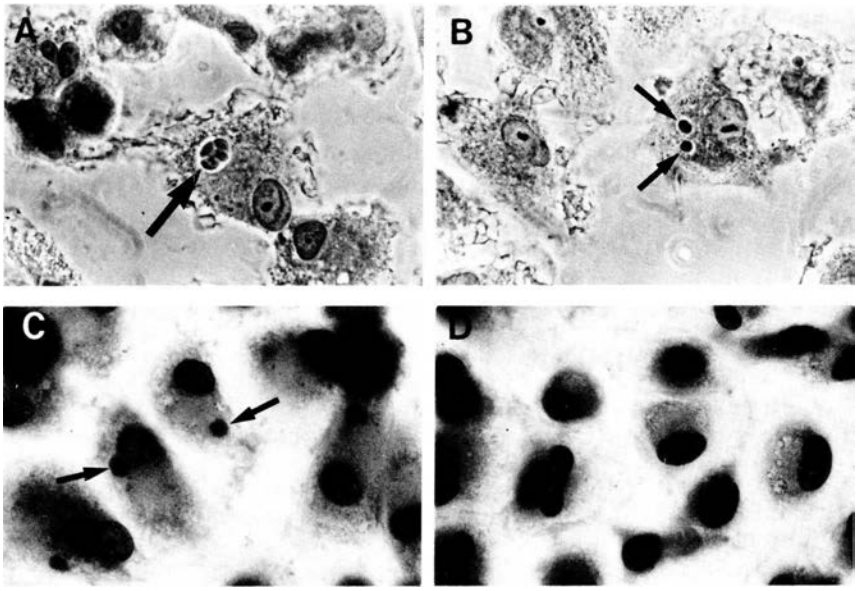

Figure 1. Phase-contrast photomicrographs of human alveolar macrophages cultivated for 72 hours prior to challenge with either *T. gondii* or *C. psittaci* (9). Micrographs show cells 20 hours after infection. (A) Unstimulated macrophages support intracellular *T. gondii* replication with four toxoplasmas per vacuole (*arrow*). (B) Lymphokine- or IFN-γ-activated macrophages, however, inhibit *T. gondii* replication. In B, neither of the two separate intracellular toxoplasmas (*arrow*) have replicated. (C) Unstimulated alveolar macrophages support *C. psittaci* replication, and show typical cytoplasmic inclusions (*arrows*) which contain numerous multiplying chlamydiae. (D) Activated macrophages inhibit *C. psittaci* replication and are largely free of inclusions (9).

patients with opportunistic infections show neither intrinsic abnormalities in intracellular antimicrobial activity nor any defect in the capacity to achieve the activated state once properly provided with effective T cell-derived activating signals. It is clear, however, that the AIDS T cell defect extends to the failure to secrete antigen-induced macrophage-activating lymphokines, in particular IFN-γ, thus rendering macrophages persistently susceptible to and entirely unable to contain newly acquired or reactivated infections caused by intracellular pathogens. In all likelihood, the failure to generate IFN-γ (or IL-2) in patients with fully established AIDS largely reflects an absolute deficiency of $T4^+$ helper cells; in some patients, however, qualitative defects in $T4^+$ cell function and/or perhaps abnormalities more proximal to $T4^+$ cell activation (e.g., accessory cell function) may also be involved. Irrespective of the mechanism responsible for absent secretion of T cell lymphokines, it is clear that macro-

phage activation is grossly impaired or entirely fails to occur in AIDS and that this acquired immunodeficiency is persistent, progressive, and ultimately accompanied by a uniformly fatal outcome once expressed clinically by the development of an opportunistic infection. Given this background, attempts to therapeutically or prophylactically induce macrophage activation in vivo by administering agents such as IFN-γ are appropriate from both a clinical as well as a scientific standpoint.

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Interferons As Components of the Host Response to Nonviral Infection

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Interferon Classes

Interferons (IFNs) are induced proteins that are characterized by their ability to render cells resistant to viral replication (antiviral activity). Three antigenically distinct classes (α, β, γ) of both murine and human IFNs are recognized (1). The IFNα and IFNβ classes are stable at low pH; however, IFNγ loses antiviral activity upon acidification. The murine IFN classes appear to be analogues of the respective human IFNs, based on amino acid sequence homologies (2–7). The human IFNγ gene is located on chromosome 12 (8) and encodes for a mature peptide of 146 amino acids (2), whereas the murine IFNγ peptide consists of 136 amino acids (3). Twelve subtypes of human IFNα proteins have been identified (9), and recombinant DNA studies indicate the existence of at least 24 human IFNα genes and pseudogenes. Multiple subtypes of murine IFNα molecules have also been reported (10). In contrast, only one human IFNβ gene has been cloned and sequenced (11–13), but there is evidence suggesting that more than one IFNβ gene exists (14).

The cellular origin of IFNγ appears to be primarily T lymphocytes (15), although natural killer cells have also been reported capable of synthesizing IFNγ (16). Sensitized T lymphocytes secrete IFNγ after specific antigenic stimulation (15). It has also been shown that polyclonal T cell mitogens can induce nonimmune T cells to produce IFNγ (17, 18). Human IFNβ is produced by cells of nonlymphoid origin, whereas human peripheral blood leukocytes synthesize IFNα in response to viral stimulation (19). In contrast to the human situation, murine nonlymphoid cells, including macrophages, produce mixtures of IFNα and IFNβ (IFNα/β) when stimulated with a variety of IFN-inducing agents (20–23).

Multiple Biological Activities of Interferons

Although initially characterized by their ability to evoke a cellular state of antiviral resistance, IFNs can mediate other activities that could con-

Table I. Some Biological Activities of Interferons

Activities common to all IFN classes
Induction of antiviral state
Induction of 2′ 5′ oligoadenylate synthetase
Induction of protein kinase
Inhibition of cell growth
Activation of natural killer cells
Activation of cytotoxic T lymphocytes
Induction of class I histocompatibility antigens
Inhibition of intracellular mulitplication of nonviral microorganisms
Activities exhibited exclusively or preferentially by IFNγ
Induction of class II histocompatibility antigens (Ia or DR)
Induction of immunoglobulin Fc receptors on macrophages
Activation of macrophages for tumor cell cytotoxicity and antimicrobial activity
Induction of myelomonocytic differentiation
Stimulation of IL-1 (?) and IL-2 synthesis
Stimulation of immunoglobulin production

ceivably affect the expression of resistance to viral and nonviral pathogens; such activities include *(a)* inhibiting cell division (24–26), *(b)* suppressing the intracellular proliferation of nonviral pathogens (27–30), *(c)* regulating the generation and/or expression of immunity (31–34), *(d)* effecting changes in the molecular composition of cell membranes (35–38), and *(e)* activating macrophages (39–44). Listed in Table I are some biological activities common to all IFNs, as well as those activities which appear to be preferentially, or exclusively, mediated by IFNγ. Biochemical studies are just now beginning to yield information that may eventually explain, at the molecular level, the basis for the differential biological activities between IFNγ and the other IFNs. IFNγ interacts with a receptor which is distinct from that to which the other IFNs bind (45, 46). After the initial interactions of IFNs with their respective receptors, striking quantitative and qualitative differences are found in the intracellular peptides induced by the IFNs (47). In addition to those peptides induced by IFNα and IFNβ, IFNγ induces the synthesis of an additional 12 peptides. These

differences between IFNγ and other IFNs, as well as the fact that IFNγ is normally produced by sensitized T cells during the expression of cellular immunity to both viral and nonviral antigens, collectively suggest that the primary function of IFNγ may be one other than eliciting a cellular state of antiviral resistance.

Interferon Induction by Nonviral Agents

Interferon was originally described almost 30 years ago as being produced both in vitro and in vivo during viral infection (48, 49). However, IFN synthesis can also be induced by nonviral agents. Stinebring and Youngner (50) reported that shortly (2 hours) after the intravenous injection of gram-negative bacteria, IFN was detected in the serum of mice, and Ho (51) reported a similar finding in rabbits. Subsequent studies established that endotoxin appeared to be the component of gram-negative bacteria that was responsible for the appearance of IFN in the serum. Other investigators found that animals produced IFN in response to many different bacteria including *Chlamydia* (52), *Rickettsia* (53), and *Mycoplasma* (54), as well as in response to higher-order organisms, such as fungi (55) and protozoa (56, 57). The IFN induced by these different agents characteristically retained antiviral activity after prolonged periods of acidification, a property of viral-induced IFN originally described by Isaacs and Lindenmann (48, 49). However, in 1970, Stinebring and Absher (58) reported that an acid-labile serum IFN was produced by *Mycobacterium tuberculosis*-infected mice after intravenous infusion of mycobacterial antigen (purified protein derivative). This finding confirmed an earlier report made by Wheelock (17) of the existence of an acid-labile IFN (IFNγ). Subsequent studies established that a wide variety of antigens (viral and nonviral), which elicited T cell-mediated immune responses, could induce IFNγ synthesis in either animals sensitized to these antigens, or in lymphocyte cultures established from these animals.

Modulation of the IFN-producing Potential of the Host by Bacteria

After the discovery that IFN could be induced by the intravenous injection of certain bacteria, it was found that bacteria could also alter the capacity of the host to produce IFN in response to IFN-inducing agents. Investigators reported a state of hyporesponsiveness to IFN-inducing agents developed shortly after the intravenous injection of gram-negative bacteria or endotoxin (59–61). This hyporesponsiveness to repeated stim-

ulation with either the same or different IFN-inducing agents decayed slowly. Other studies showed that the composition of the host's microflora could also alter IFN responses. Gnotobiotic mice possessing a monoflora of gram-negative bacteria, when challenged with virus, produced less serum IFNα/β than conventional control animals (62). On the other hand, certain bacteria that typically evoke cell-mediated immune responses, such as mycobacteria (60) or *Propionibacterium acnes* (63), were reported to enhance the potential of the host to produce IFNα/β.

Our original interest in the relationship between bacterial infection and the potential of the host to produce IFN evolved from a need to produce substantial quantities of IFNγ for biochemical studies. Prior to the availability of recombinant murine IFNγ, the methodology for the production of IFNγ involved incubating lymphoid cells with different polyclonal T cell mitogens. Over a period of 6 months in 1981, a number of different procedures were tried using various polyclonal T cell mitogens to induce IFNγ synthesis in spleen cell cultures. However, the resulting IFNγ titers rarely exceeded 100 U/ml. But then, as serendipity would have it, spleen cell cultures were established from mice with an ongoing sublethal infection with the gram-positive facultative intracellular bacterium, *Listeria monocytogenes*, and then these cultures were incubated with phytohemagglutinin (PHA). In this experiment it was found that the 24-hour PHA-induced IFNγ titers in the culture supernatants were at least 20 times higher than the IFNγ titers in culture supernatants from similarly treated normal spleen cells (18). Additional studies showed a striking temporal association between the enhanced potential for IFNγ synthesis and the development of anti-*Listeria* immunity, in that both developed and decayed in unison. This relationship is clearly illustrated in Figure 1, where it can be seen that the peak of anti-*Listeria* immunity (day 6) coincides with the greatest potential for PHA-induced IFNγ synthesis. Through a series of studies using specific antibodies and complement to deplete different spleen cell populations, it was determined that PHA induction of IFNγ synthesis in spleen cell cultures from *Listeria*-infected mice was T-cell dependent, and that T cells of the Lyt-2^- phenotype appeared to be the major source of PHA-induced IFNγ in this system. It has now been established that specific antigen stimulation, using either ultraviolet (UV)-killed or viable *Listeria* to induce 6-day *Listeria*-immune spleen cell cultures in media containing a listeriostatic concentration of the antibiotic gentamicin sulfate, results in IFNγ titers that are approximately equivalent to those obtained under optimal conditions with PHA (Table II). However, unlike PHA, the *Listeria* preparations also induce low levels of IFNα/β. Because there is evidence indicating that interleukin

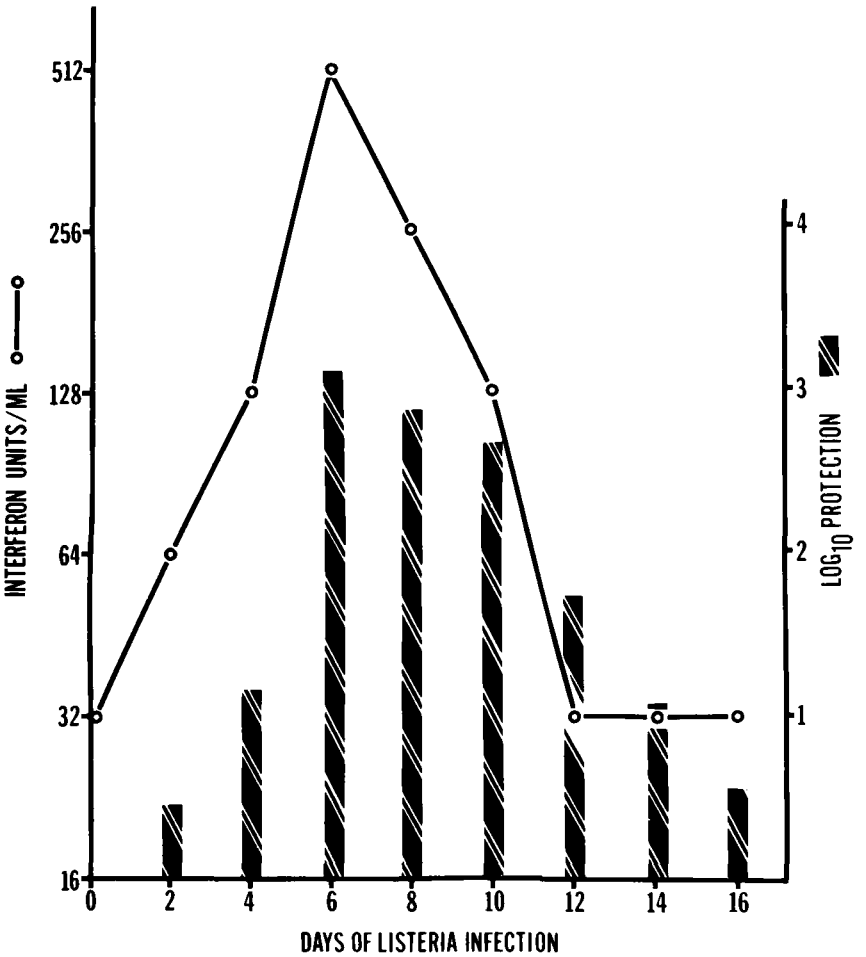

Figure 1. Development of anti-*Listeria* immunity and the capacity for enhanced IFNγ synthesis by spleen cells from *Listeria*-infected mice. At the indicated day after intravenous injection of 2 × 10^3 *Listeria monocytogenes*, pooled spleen cells from groups of five mice were either induced with 5 μg/ml of PHA for 24 hours to determine their ability to produce IFNγ in vitro (*line graph*) or tested for their ability adoptively to transfer anti-*Listeria* immunity in vivo (*bar graph*).

2 (IL-2) may be the terminal signal for IFNγ synthesis induced by either polyclonal T cell mitogens or specific antigen (64–67), cultures of 6-day *Listeria*-immune or control spleen cells were also incubated with 100 U/ml of recombinant human IL-2. The IL-2 induced low levels of IFNγ only in the 6-day *Listeria*-immune cell cultures.

Recent studies have established that mice infected with *Listeria* also acquire an enhanced potential to produce IFNα/β in response to IFN-

Table II. Induction and Antigenic Characterization of Interferons Induced by Various Agents in Cultures of Control and Listeria-immune Spleen Cells

Mode of induction	*Spleen cells*[a]	*Antiviral activity after*			
		None	*anti-α/β*	*anti-γ*	*anti-α/β* + anti-γ
		U/ml			
None	Control	<4	–	–	–
None	6-day *Listeria*-immune	4	4	<4	<4
10^7 viable *Listeria*/ml	Control	64	64	<4	<4
10^7 viable *Listeria*/ml	6-day *Listeria*-immune	512	512	8	<4
10^7 UV-killed *Listeria*/ml	Control	32	32	<4	<4
10^7 UV-killed *Listeria*/ml	6-day *Listeria*-immune	512	512	32	<4
PHA (5 μg/ml)	Control	32	32	<4	<4
PHA (5 μg/ml)	6-day *Listeria*-immune	1,024	512	<4	<4
IL-2 (100 U/ml)	Control	<4	–	–	–
IL-2 (100 U/ml)	6-day *Listeria*-immune	16	8	<4	<4

a. Spleen cells (10^7/ml) were incubated in RPMI 1640 medium, containing a listeriostatic concentration of gentamicin sulfate at 37°C. Twenty-four hours later, culture media were collected, and samples were reacted with, or without excesses of the designated anti-IFN antibodies for 1 hour, and then assayed for IFN activity.

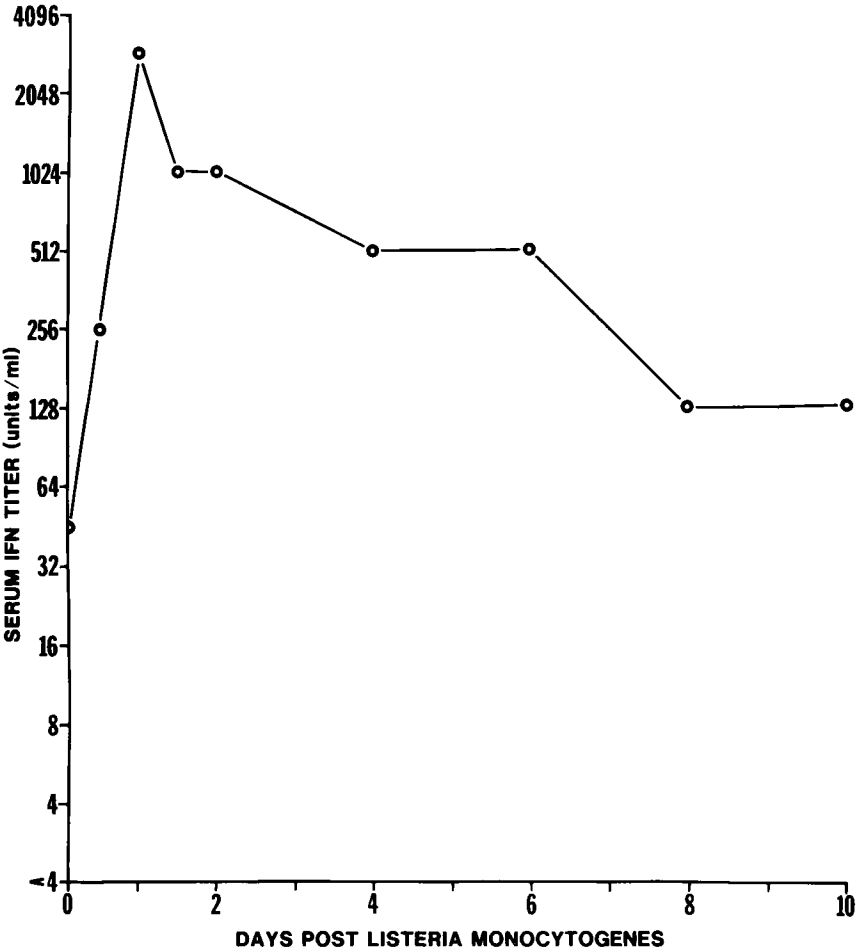

Figure 2. Endotoxin-induced serum IFN levels during *Listeria monocytogenes* infection. Serum IFN levels were measured 2 hours after intravenous injection of 25 μg of endotoxin in mice at progressive times after inoculation (0 hour) of an immunizing *Listeria* dose.

inducing agents. As early as 8 hours after the intravenous injection of 2×10^3 *Listeria*, infected mice produce five times more serum IFNα/β than normal mice after the intravenous injection of endotoxin. The results presented in Figure 2 show endotoxin-induced serum IFN levels in mice at progressive times after the initiation of *Listeria* infection. Maximum endotoxin-induced IFN titers occur 24 hours after *Listeria* inoculation, with infected mice producing 64-fold more serum IFN activity than normal mice. After 24 hours, the enhanced responsiveness of infected mice slowly declines. The kinetics of the appearance of endotoxin-induced IFN in the sera of normal mice, and mice inoculated with *Listeria* 24 hours ear-

lier, are presented in Figure 3. The highest serum IFN titers are detected in both groups 2 hours after endotoxin injection. At this time, the sera of *Listeria*-infected mice possess 32 times more IFN activity than sera of normal mice. Other studies have established that viable *Listeria* are required for the augmented IFN response of the host. Furthermore, the mechanism responsible for enhanced IFN production appears to be T cell-independent, because elevated levels of endotoxin-induced IFN were found in the sera of *Listeria*-infected mice made T cell-deficient by thymectomy followed by gamma irradiation and bone marrow reconstitution (TXB mice), as well as *Listeria*-infected congenitally athymic (nu/nu) mice. The IFN activity in the sera at the peak (2 hours) of the endotoxin-induced IFN response was determined to be exclusively IFNα/β by means of specific anti-IFN antibody neutralization assays. Thus, *Listeria monocytogenes*, like other bacteria that elicit cell-mediated immune responses (60, 63), primes the host for IFNα/β production. This priming occurs shortly after the onset of infection, and peaks 1 day prior to the time when anti-*Listeria*-sensitized T cells can be first detected.

Listeria monocytogenes-induced Interferons

Spleen cells from day 6 *Listeria*-infected mice, when placed in culture with medium containing antibiotics, produce marginal levels of IFNγ

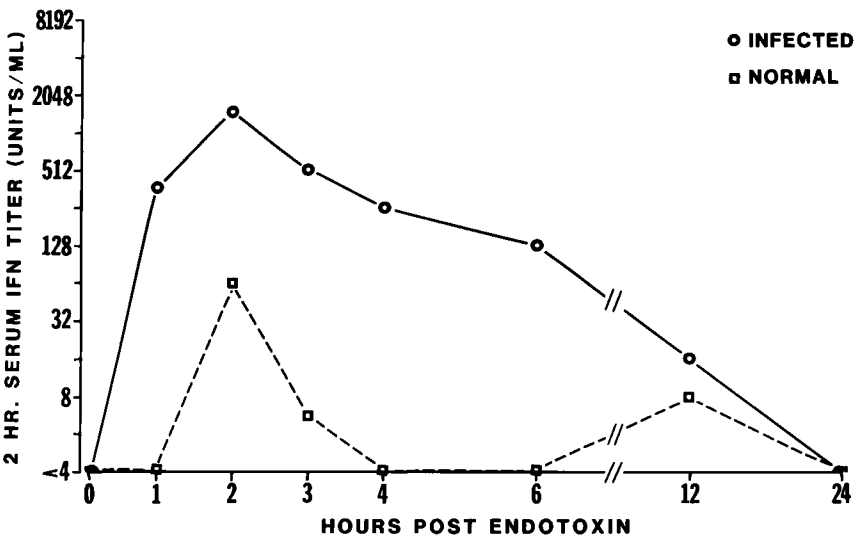

Figure 3. Kinetics of appearance of endotoxin-induced serum IFN in sera of normal and 1-day *Listeria*-infected mice. Normal and 1-day *Listeria*-infected mice were injected intravenously with 25 μg of endotoxin (0 hours), and bled at the indicated times.

(Table II). The numbers of viable *Listeria* in the spleen on the sixth day of infection is estimated to be around 200, and the low levels of IFNγ detected in the culture supernatants are presumably due to *Listeria* present within the spleen cell suspension specifically inducing an IFNγ response. Although our studies established that the greatest potential for IFNγ synthesis occurs on day 6 of infection, it is known that the expression of anti-*Listeria* immunity begins on, or shortly after, the second day of infection, when peak numbers of *Listeria* (10^5) are present in the liver and spleen. Thus, the peak of antigenic stimulation and highest levels of IFNγ produced *in vivo*, may occur prior to the peak of the anti-*Listeria* immune response on day 6. To determine whether IFNγ could be detected in the circulation at any time during listeriosis, mice were bled at progressive times after the intravenous injection of a sublethal *Listeria* inoculum, and the sera were assayed for antiviral activity. Antiviral activity was first detected in the serum 24 hours after *Listeria* injection, after which the IFN titer peaked at 48 hours (Table III). After the fifth day of infection, no antiviral activity was detected in the serum. Surprisingly, serologic analysis revealed that all the antiviral activity present in the sera during listeriosis was due to IFNα/β. A correlation exists between the numbers of *Listeria* in infected target organs (liver and spleen) and levels of IFNα/β present in the serum, inasmuch as the highest levels of serum IFNα/β

Table III. Listeria monocytogenes-induced Serum Interferon Titers in Mice at Progressive Times of Infection

Time of infection[a]	*Serum interferon titer*
hours	*U/ml*
0	<4
18	<4
24	4
48	32
72	8
96	4
120	<4
144	<4

a. At 0 hour, mice were injected intravenously with an immunizing dose (4×10^3) of *Listeria monocytogenes*.

are detected at approximately the same time peak *Listeria* numbers are reached in the target organs on day 2 of infection. In support of this relationship, it was found that by increasing the inoculum from 4×10^3 to 10^5 or 10^6 organisms resulted in the appearance of serum IFNα/β at 18 and 12 hours, respectively. The inability to detect IFNγ in the systemic circulation at any time during infection, indicates that IFNγ is probably produced by sensitized T cells only locally within infectious foci.

Functional Analysis of Interferons in Resistance to Listeria monocytogenes

The results of our studies have established that during the course of a sublethal *Listeria monocytogenes* infection all three IFN classes are produced, and that the potential of the host to produce the different IFNs is enhanced. These findings raise fundamental questions as to the possible functions of these antiviral molecules in nonspecific and specifically acquired resistance to a nonviral pathogen.

The response of mice to *Listeria monocytogenes* offers an excellent model for studying the possible roles for IFNs in preimmunity resistance and acquired specific resistance to a facultative intracellular pathogen. The expression of anti-*Listeria* resistance is dependent upon the generation of activated macrophages capable of expressing microbicidal function (68). In specifically acquired resistance, macrophage activation is mediated by an acquired population of specifically-sensitized T cells, which are first detected in the spleen on the second day of infection (69). Based on passive transfer experiments, it has been repeatedly shown that peak numbers of *Listeria*-immune T cells are generated on the sixth day of infection, after which these cells rapidly decrease in number, as *Listeria* is eliminated from the tissues (69). The results of a study that compared resistance to *Listeria* infections in T cell-intact (nu/+) and T cell-deficient nude (nu/nu) mice, suggest that two T cell-independent mechanisms of anti-*Listeria* resistance are expressed before the generation of T cell-mediated anti-*Listeria* immunity (70). After intravenous inoculation, *Listeria* is taken up primarily by spleen and liver macrophages, and during the next 12 hours a substantial proportion of the inoculum is destroyed. It was shown that this initial kill of bacteria is mediated by radioresistant cells, thought to be resident tissue macrophages (70). After the first 24 hours of infection, the host develops a radiosensitive T cell-independent mechanism that functions to limit progressive bacterial growth of organisms that survive the initial kill. Both mechanisms of resistance are expressed in nude mice. Unlike the acute short-lived *Listeria* infection

in T cell-intact mice, listeriosis in nude mice after the second day of infection is characterized by the persistence of a chronic state of infection, that presumably fails to progress because of the anti-*Listeria* actions of macrophages (70–73). The macrophages mediating this T cell-independent resistance after 48 hours are believed to be freshly recruited from the blood into infective foci. In nude mice, the number of *Listeria* in the liver and spleen remains at approximately 10^5 organisms/organ for upwards to 3 weeks. After this time, progressive *Listeria* growth causes death (70).

Evidence suggesting that IFNs produced during listeriosis may function in host resistance comes from studies where crude preparations of IFNα/β or IFNγ were found to protect mice from a lethal dose of *Listeria* (Table IV). Two potential anti-*Listeria* effects of IFNs are currently under study in this laboratory. First, histologic examination of infected livers has revealed large numbers of *Listeria* within cells (hepatocytes) that are normally not phagocytic (74) and therefore, in view of the demonstrated inhibitory effect of IFNs on the intracellular multiplication of obligate and facultative intracellular bacteria (44–47), it is possible that IFNs produced during listeriosis may contribute to host resistance by suppressing intracellular proliferation of *Listeria*. Second, the demonstration of a temporal relationship between the development of T cell-mediated anti-*Listeria* immunity and the enhanced potential for IFNγ synthesis (18), as well as the results of studies establishing that IFNγ preparations activate the parasiticidal and tumoricidal mechanism of macrophages, strongly suggest that IFNγ may affect macrophage activation during listeriosis.

Table IV. Protective Actions of Interferons against a Lethal Listeria Challenge

Treatment[a] 24 hours prior to infection[b]	*Number of mice surviving on day of infection*				
	3	*4*	*5*	*6*	*14*
None	5/5	3/5	0/5		
PBS	5/5	0/5			
IFNα/β	5/5	5/5	5/5	5/5	5/5
IFNγ	5/5	5/5	5/5	5/5	5/5

a. Intravenous injection of 0.2 ml of phosphate-buffered saline (PBS); 0.2 ml of IFNα/β (2,000 U); or 0.2 ml of IFNγ (2,000 U).

b. One day after the treatments, mice were inoculated with 5 LD_{50} of *Listeria monocytogenes*.

However, to conclude that an IFN functions in any aspect of resistance requires the use of homogeneously pure IFN, or a specific anti-IFN antibody which can block the possible IFN-mediated effect under study.

The development of the *Listeria*-immune spleen cell system for the production of large quantities of PHA-induced high-titered IFNγ enabled both the analysis of certain physicochemical properties of this lymphokine, as well as its purification. The major IFNγ component in these spleen cell culture supernatants was found to have an acidic isoelectric point (pH 5.6), and to bind specifically to immobilized concanavalin A (75), indicating its glycoprotein nature. This IFNγ was determined to have a molecular weight of 38,000 under nondenaturing conditions (18). However, under denaturing and reducing conditions the molecular weight was estimated to be 20,000 (76), which suggests that monomeric units aggregate to form oligomers. The results of these characterization studies greatly facilitated purification efforts. A partially purified IFNγ (10^6 U/mg of protein) proved to be a potent immunogen in rats (77). B cells from an immunized rat were fused to mouse myeloma cells (P3U-1) and a stable hybridoma (R4-6A2) was cloned that secretes potent rat anti-IFNγ IgG-neutralizing monoclonal antibody (MAb) (44). This MAb, when used as an affinity immunoadsorbent, has recently allowed the single-step purification of IFNγ from crude lymphokine preparations.

The specificity of the rat anti-IFNγ MAb was tested against a number of different IFN preparations. The MAb neutralized the antiviral activity of all murine IFNγ preparations tested, including a preparation of recombinant DNA murine IFNγ (44). Because the recombinant IFNγ is not glycosylated, its neutralization by the MAb indicated that the epitope recognized by the MAb resides within the polypeptide moiety of the IFNγ molecule. In contrast, the antiviral activities of murine IFNα/β and human IFNγ were not neutralized by the MAb. The amount of MAb required to neutralize murine IFNγ was found to be directly proportional to the titer of antiviral activity. In Figure 4, a dose-response curve for the MAb neutralization of homologous (murine) and heterologous (rat) IFNγ antiviral activities reveals a linear relationship between the quantity of MAb required for neutralization and amount of IFNγ (antiviral activity). Because rat fibroblast cells are twofold less sensitive than murine cells to murine IFNγ, twice as much MAb was required to abate an equivalent amount of IFNγ antiviral activity. The ability of PHA-induced spleen cell culture supernatants to inhibit cellular proliferation (anticellular action) was shown also to be due to IFNγ through the use of the anti-IFNγ MAb. Cultures of L929B cells were incubated with 100 antiviral units per milliliter of a lymphokine preparation and cultures

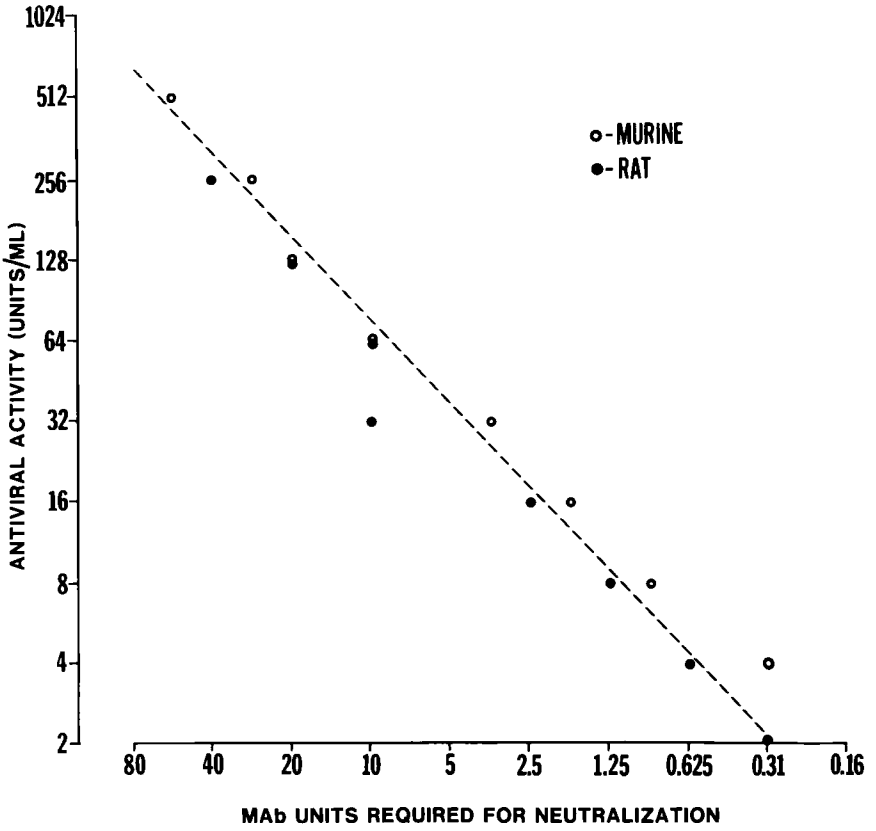

Figure 4. Linear relationship between quantity of monoclonal anti-IFNγ IgG (MAb) required for neutralization and IFNγ antiviral activity on rat and murine cells.

were treated with increasing MAb concentrations. Five days later, the amount of antiviral activity in the culture media and the number of cells in the variously treated groups were determined (Figure 5). Cell growth was inhibited in those cultures treated with insufficient quantities of MAb to neutralize IFNγ, as assessed by the residual amounts of antiviral activity remaining in the media, whereas cell growth progressed unabated in lymphokine-treated cultures, where all antiviral activity was neutralized by the MAb.

The anti-IFNγ MAb has also proven instrumental in establishing IFNγ as the component present in lymphokine preparations that activates cultured macrophages to express enhanced tumoricidal activity (44). The demonstration that IFNγ enhances the tumoricidal actions of macrophages was established in two ways. The first approach was to specifically

remove IFNγ from the lymphokine preparation by using immobilized MAb as an affinity immunoadsorbent. The unbound lymphokine fraction was unable to elicit the enhanced macrophage tumoricidal activity that was observed after incubation of macrophages with the unfractionated preparation. The MAb also neutralized the ability of lymphokine preparations to activate the tumoricidal activities of macrophages; however, the degree of neutralization differed from that for IFNγ-mediated antiviral activity (44). It was observed that, based on relative antiviral activity, the neutralization of IFNγ-induced macrophage tumoricidal activity required substantially more MAb than that required to neutralize IFNγ antiviral activity. The direct proportionality observed between the quantities of MAb required for neutralization of given amounts of IFN antiviral activity was not observed for IFNγ-mediated macrophage activation as measured by cytolysis of tumor target cells.

The availability of the neutralizing anti-IFNγ MAb raises the possi-

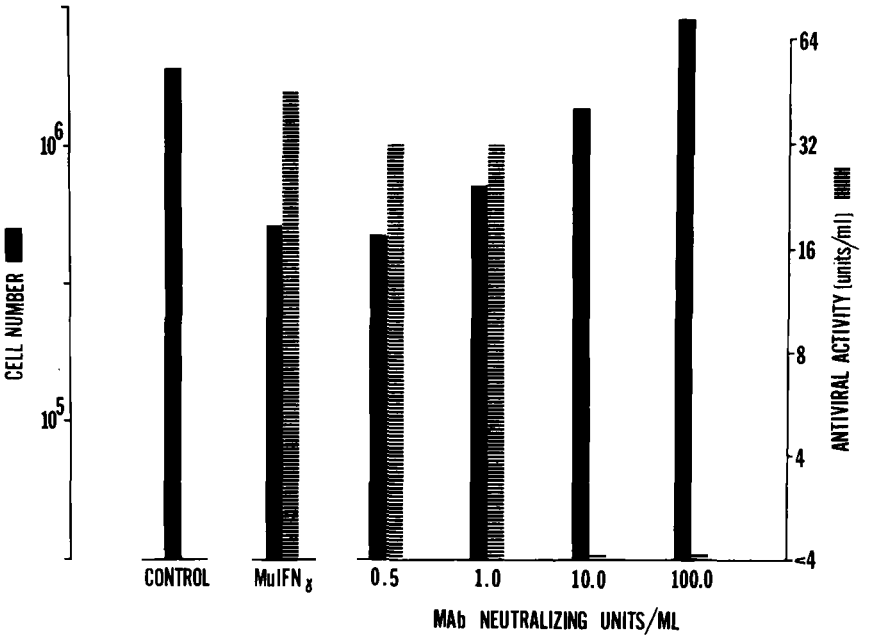

Figure 5. Anti-IFNγ MAb neutralization of anticellular activity (growth inhibition) of IFNγ. Murine L929 cells (5×10^5) were seeded in culture dishes (60-mm) containing either medium alone (control), medium containing 100 IFNγ U/ml, or in medium containing 100 U of IFNγ with the designated anti-MAb concentrations. Five days later, the culture supernatants were assayed for IFNγ antiviral activity, and the number of L929 cells were determined in the variously treated cultures by cell counting. One unit of MAb neutralizes 10 U of IFNγ antiviral activity.

bility that it could be used in vivo to establish whether IFNγ, synthesized during the course of T cell-mediated immune responses, is indeed an essential component in the expression of immunity. During the course of a sublethal *Listeria monocytogenes* infection, *Listeria*-sensitized T cells cause the activation of the antimicrobial actions of macrophages, which in turn eradicate the pathogen. Thus, if IFNγ produced by sensitized T cells affects physiological changes (activation) that enable macrophages to resolve the infection through enhanced listericidal actions, the blocking of IFNγ function by administration of excess anti-IFNγ MAb should result in an exacerbation of infection. Therefore, the infection might become progressively lethal, or it may become chronic, as in athymic nude mice. Conversely, the administration of IFNγ into chronically infected nude mice would also determine whether this lymphokine activates the listericidal actions of macrophages. Macrophage activation would be expected to cause partial or total elimination of *Listeria* in the livers and spleens of IFNγ-treated nude mice.

Interferons inhibit intracellular multiplication of *Shigella* (28), *Chlamydia* (29), and *Rickettsia* (30). Because *Listeria monocytogenes* can proliferate in both phagocytic and nonphagocytic cells, the IFNs produced during listeriosis may suppress intracellular *Listeria* multiplication and thus limit the number of bacteria in the host until an effective cell-mediated immune response is mounted to resolve the infection. Studies have been initiated to examine the possible effects of IFNs on intracellular *Listeria* proliferation in murine embryo fibroblast cells (MECs). Incubation of MEC monolayers for 2 hours with *Listeria* at a multiplicity of 10 *Listeria* per cell, results in 1–5% of the cells becoming infected, with one to three bacteria per cell. After this initial infection procedure, the MECs are extensively washed and reincubated in medium containing a concentration of the antibiotic gentamicin sulfate, which inhibits only extracellular bacterial multiplication. Enumeration of intracellular *Listeria* is carried out by lysing the MECs with deoxycholate, and plating serial dilutions of lysate. Intracellular and extracellular growth curves for *Listeria* are presented in Figure 6. Intracellular *Listeria* numbers increase exponentially, with an approximate doubling time of 2.5 hours, whereas the doubling time for extracellular *Listeria* in culture medium, without MECs or antibiotic, was estimated to be approximately 30 minutes. The micrographs in Figure 7 show *Listeria*-infected MECs at 2 (panel A) and 24 (panel B) hours after the initial infection procedure. After the initial infection of MECs, foci of infected cells are produced as the result of subsequent infection of contiguous cells. The size of infectious foci progressively increase over time, with the infection eventually resulting in the

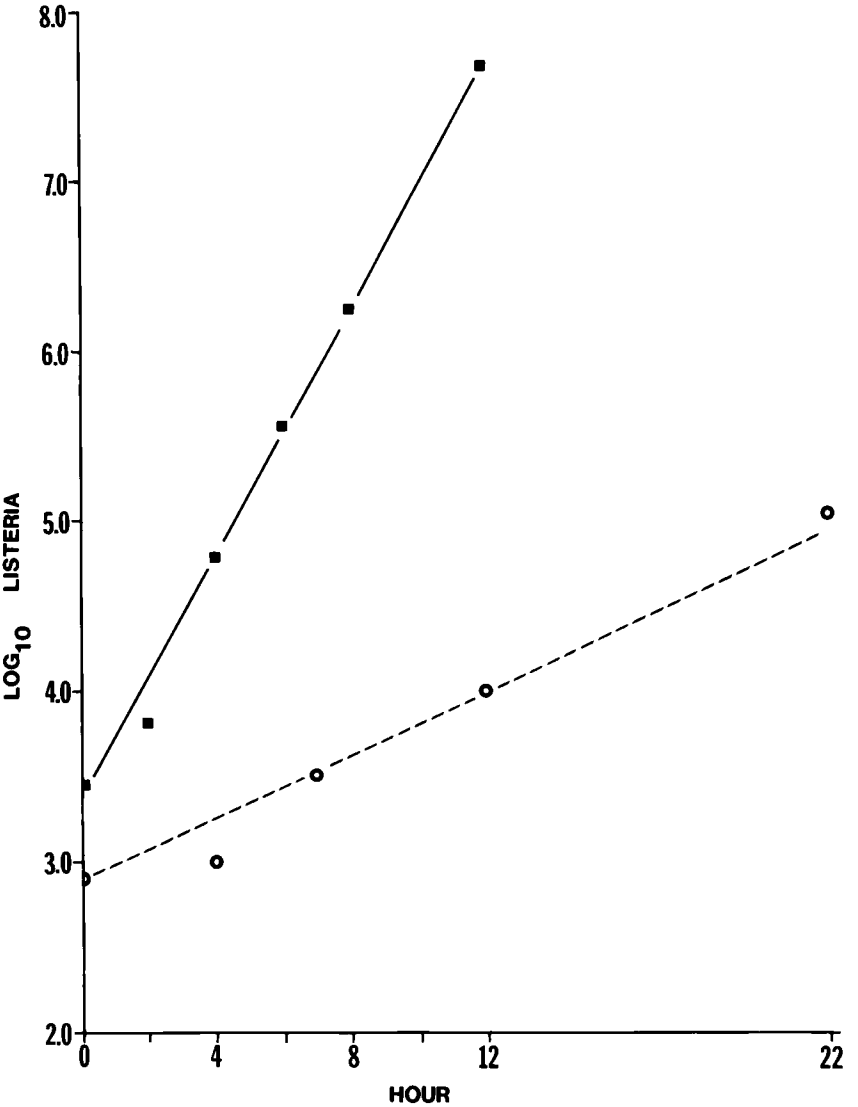

Figure 6. Intracellular (○) and extracellular (■) *Listeria monocytogenes* growth curves. Extracellular proliferation was determined by inoculating replicate 35-mm dishes containing 2 ml of antibiotic-free MEC growth medium with 3×10^3 *Listeria*. The number of *Listeria* at the designated times after inoculation was determined by plating the media from replicate cultures on trypticase-soy agar. Details for the enumeration of intracellular *Listeria* are presented in the text.

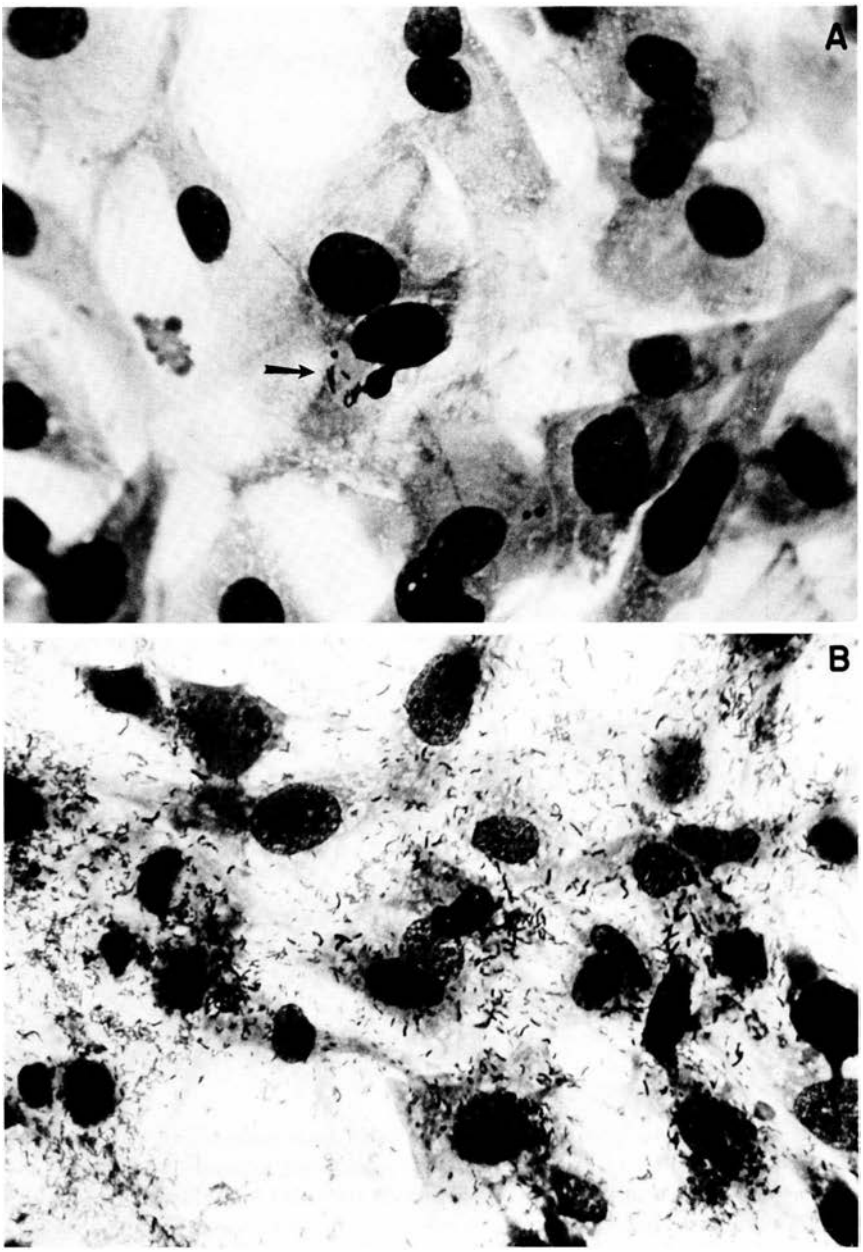

Figure 7. Listeria monocytogenes infection of MECs. (A) An infected MEC (*arrow*) surrounded by uninfected cells 4 hours after initiation of infection in vitro. (B) *Listeria monocytogenes* infection 22 hours later, in an area where the initial number of MECs infected was greater than that evident in panel A (×270).

destruction of the MEC monolayer (Figure 7B). Preliminary studies have also examined the effect of IFNs on the course of *Listeria* infection in MECs. The MEC cultures were incubated with either IFNα/β or IFNγ for 24 hours, after which they were infected with *Listeria*. Enumerations of intracellular *Listeria* in treated and control cultures were carried out 24 hours later. The numbers of *Listeria* in the cultures treated with either IFN preparation were greatly reduced in comparison with the *Listeria* numbers in the untreated MEC cultures. These findings indicate that IFNs either inhibit the intracellular multiplication of *Listeria* and/or the initial internalization of bacteria. Studies are underway to distinguish between these possibilities.

It has also been found that IFNα/β is synthesized by MECs during the intracellular proliferation of *Listeria*. This finding may indicate that nonphagocytic cells infected with *Listeria* may be a source of IFNα/β detected in the serum of infected mice during the exponential growth of the pathogen. However, it should also be stated that, in regards to potential in vivo cell sources of *Listeria*-induced IFNα/β, macrophages isolated from *Listeria*-infected organs, also produce these molecules when placed in culture.

Summary

Immunity to the facultative intracellular bacterium, *Listeria monocytogenes*, is a well-studied model of cellular immunity, wherein *Listeria*-sensitized T cells cause activation of the bactericidal mechanisms of macrophages. During an immunizing infection with *Listeria*, the host acquires a greatly augmented capacity to produce all three antigenically distinct classes (α, β, γ) of interferon. Moreover, during the initial preimmune phase of infection, IFNα and IFNβ (IFNα/β) appear in the circulation, whereas the lymphokine IFNγ is produced later and only within infected organs during the expression of specific anti-*Listeria* immunity. The most obvious function of these antiviral molecules would be to protect the compromised host from a second infection with virus, however, in view of the multiple activities frequently ascribed to IFNs, it is conceivable that these molecules may function in nonspecific and/or specific host resistance mechanisms to nonviral pathogens.

Presented in this chapter is a brief review of IFN induction by bacteria, and of the reported effects of bacterial infection on the responsiveness of infected hosts to IFN-inducing agents. Particular emphasis is given to murine listeriosis as a model in which to elucidate the possible nonantiviral functions of each IFN class. One approach to determine whether

an IFN synthesized during infection is involved in an aspect of resistance would be to block the IFN-mediated effect using specific neutralizing anti-IFN antibodies. Thus, if a *Listeria*-induced IFN is involved in an aspect of resistance, then its neutralization by antibody should result in an exacerbation of infection. Conversely, the administration of a homogeneously pure IFN at different times of infection would be expected to enhance resistance, as well as reveal the stage of infection where the IFN mediates its function. Such experimental protocols are now feasible owing to both the development of hybridomas that secrete specific monoclonal neutralizing anti-IFN antibodies, and the successful purification of substantial quantities of each murine IFN.

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Response to Tumors and Immunotherapy

Tumor-specific Antigens and Immunologic Resistance to Cancer

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Introduction

Unequivocal evidence for the existence of tumor-specific antigens and host resistance to transplanted cancer cells is found in the example of ultraviolet (UV) light-induced cancers of mice. This model also clearly demonstrates various mechanisms that render strong host resistance completely ineffective. Finally, results obtained with this model demonstrate the need for reevaluating some of the earlier results obtained in other experimental tumor systems. The subsequent review will, therefore, focus on recent work in the UV-induced tumor model but refer to other models where appropriate.

The model of UV-induced tumors (1) has several advantages. First, most (i.e., more than 90%) of these tumors have strong tumor-specific antigens and are rejected regularly when transplanted into normal syngeneic hosts even without any prior immunization (Figure 1). These tumors have been induced in pathogen-free mice of defined genetic background. Large numbers of concurrently isolated tumors are available as control so that the genetic drift of the mouse strain with time can be detected immediately by the loss of tumor specificity. Because these tumors are not readily transplantable into normal mice, they have usually been preserved in their original phenotype. Fragments of the primary tumors have been frozen and tumor cells have been adapted to culture and cloned immediately from the primary tumor or after few passages in TXB or nude mice. This avoids the possibility of extensive antigenic changes resulting from immune selection that can easily occur unrecognized after prolonged or even a single passage in normal mice.[1]

1. Some investigators argue that "spontaneous" murine cancers represent an experimental model that more closely simulates human malignancy (2, 3). Though spontaneous cancers have been said to be nonantigenic (4–6), studies with these tumors are complicated by the fact that almost all have been serially transplanted (4–6) and may, therefore, represent immunoselected non-, or least, antigenic variants. Finally, it is not clear how spontaneous murine tumors may be related to human cancers, many of which are induced by chemical or physical carcinogens (8).

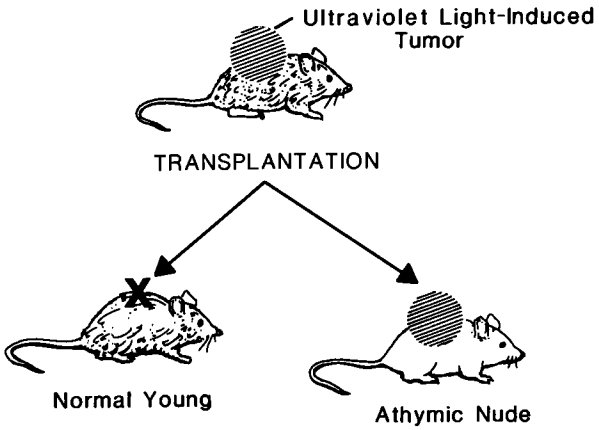


Figure 1. Strong antigenicity of murine UV light-induced skin tumors as detected by transplantation. Over 90% of such tumors are regularly rejected by normal syngeneic mice without prior immunization although these tumors do grow progressively in athymic nude mice.

Cancer induction, development, and growth clearly involve interactions among the carcinogen, the tumor cell, and the immune system (Figure 2). The model of UV-induced tumors discussed below demonstrates clearly the importance and complexity of such interactions. For example, UV light not only induces skin cancers, but it also suppresses effectively the immune system (for review, see Reference 1). Furthermore, cancer cells of UV-induced tumors not only stimulate the immune system to generate protective immunity, but can also induce profound immune suppression (9). The immune system, on the other hand, fails under certain conditions to eliminate effectively all UV-induced cancer cells. Instead, it selects for antigenic tumor cell variants that have an increased malignant potential and an increased capability to suppress the immune system (10–13).

Effects of the Immune System on the Cancer Cell

Immune Defenses of the Normal Host against Cancer and the Selection of Tumor Variants

Different types of immune cells or antibody can destroy cancer cells in vitro (14–20) or after adoptive transfer in vivo, but the different mechanisms demonstrated in vitro may or may not be relevant to understanding the mechanism of tumor rejection by the intact host. If lymphoid cells have an important tumoricidal function, then tumor cells must circum-

vent such effector cells before progressive tumor growth can occur. Neoplasms frequently develop as a clone from a single transformed cell. During the continuous growth of the clone, phenotypic variants continuously may arise as a consequence of the genetic instability that malignant cells acquire as a result of malignant transformation (21). If the phenotypic variants have a selective growth advantage because of escape from immunologic or other homeostatic control mechanisms, then these variants become the precursors of a new subpopulation that becomes dominant. The phenotypic changes should then indicate which kind of selection processes have occurred, and (the type of phenotype change should) give an insight into the relative importance of different naturally occuring defense mechanisms that may function in normal immunocompetent mice. This type of analysis is analogous to that done by a scientist who deduces the action of an antibiotic from the type of change found in the bacterium that has become drug resistant.

Ultraviolet light-induced tumors provide an excellent model for such studies (1). These tumors have strong tumor-specific antigens and are regularly rejected by syngeneic mice without prior immunization, but these tumors can grow in mice with immune deficiency present in various conditions (Figure 3). Only in very rare instances (less than 1% of the recipients), such UV-induced regressor tumors escape rejection by normal young mice by undergoing some heritable change. We have reisolated these rare escape variants of the regressor tumor 1591 and tested them in vitro for sensitivity to cytolytic T cells, activated macrophages (Mϕ), and natural killer (NK) cells (10). Furthermore, the reisolated

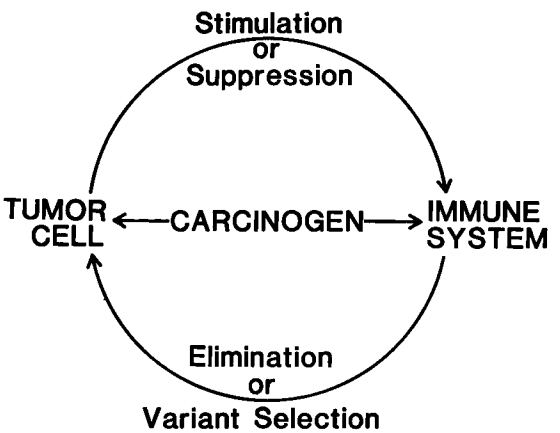


Figure 2. Scheme of interactions among carcinogen, the target cell, and the immune system as they occur in the model of murine UV-induced tumors.

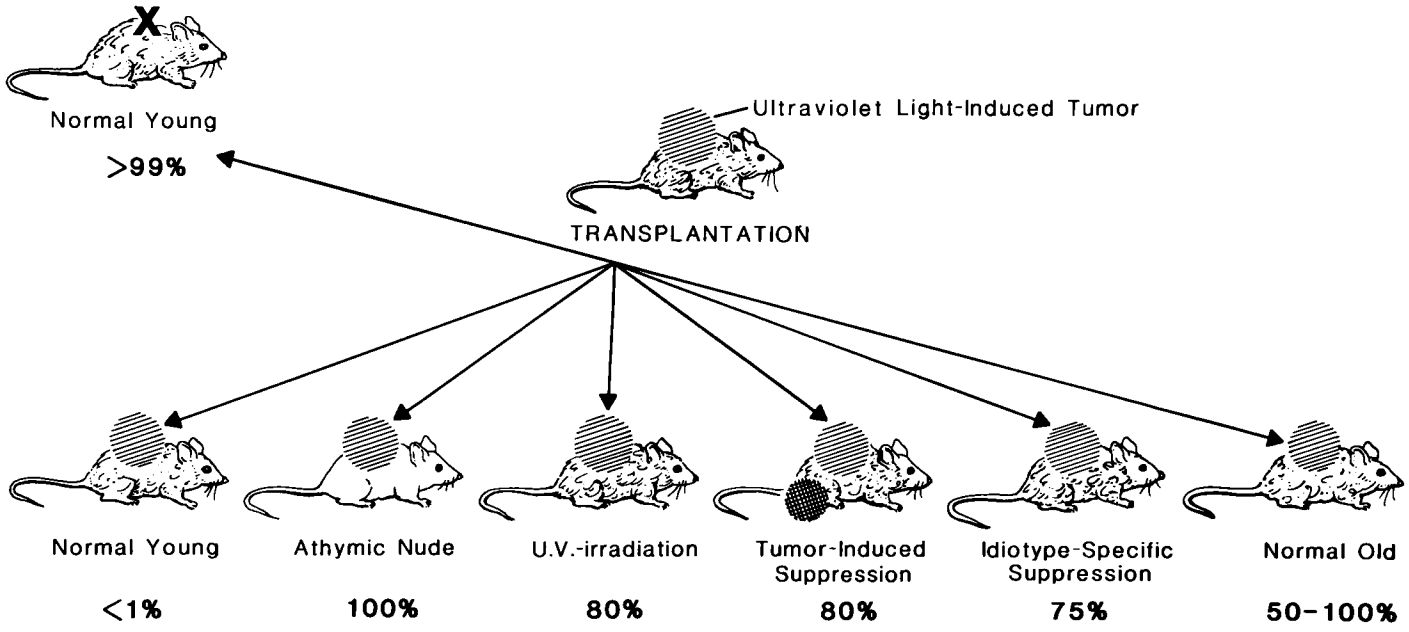


Figure 3. Growth characteristics of ultraviolet light-induced skin tumors when transplanted into mice of the strain of tumor origin. Often such tumors are strongly antigenic (e.g., tumor 1591), and such tumors are then rejected regularly by normal syngeneic mice without any prior immunization (1). Only on rare occasions do progressor variants arise after tumor transplantation into normal mice (10). However, the tumors will grow regularly in nude mice or mice immunosuppressed by UV irradiation (11, 22–24). Mice bearing a UV-induced tumor are also immunosuppressed in that they fail to reject a UV-induced regressor transplanted at a separate site (9). Selective idiotype-specific immune suppression of the tumor-specific T cell response also appears sufficient to permit the outgrowth of these highly immunogenic tumors (25–27). Finally, advanced age also makes the normal mice accept transplanted UV-induced tumors (26, 28, 29).

Table I. Absence or Presence of Immunoselection Dependent upon Host Environment

Environment	*T cell immunity*	*Progressive tumor growth*	*Phenotype of reisolated tumor*				*Reference*
			Antigenic variants	*Progressor variants*	*Mϕ-resistant variants*	*NK-resistant variants*	
	%						
Normal	Yes	<1	100	100	100	0	10, 12
Anti-idiotypic suppression	No	75	0	0	0	—[a]	11, 12
Tumor burden	No	80	0	0	0	—	9
Advanced age (24 months)	No	100	0	0	0	—	29
Athymic nude	No	100	0	0	0	—	11, 12
Ultraviolet irradiation	Partial	80	100	70	80	—	11
Selection in vitro with T cells	Yes	66	100	66	0	—	13
Selection in vitro with activated Mϕ	No	0	0	0	100	0	12

a. Not done.

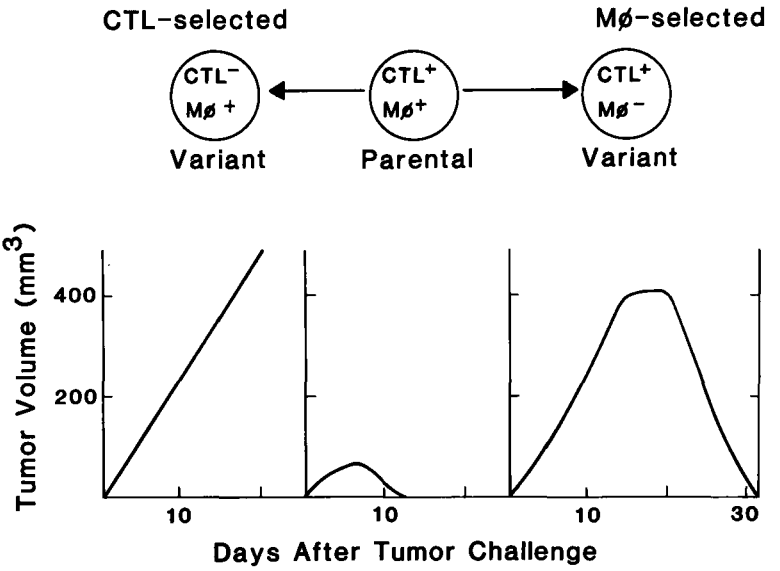


Figure 4. Differences in growth characteristics of in vitro selected 1591 variants when transplanted into normal syngeneic mice. Variants selected for resistance to activated Mϕ ($M\phi^-$ CTL^+) grow initially much more rapidly than the parental tumor ($M\phi^+$ CTL^+) but are eventually rejected (12), whereas the converse variants that have lost the CTL-recognized rejection antigen (CTL^- $M\phi^+$) grow progressively and kill the host (13). Although these variants are still susceptible to Mϕ already activated, they may fail in vivo to trigger T cells to release lymphokine(s) needed for Mϕ activation. The variants and the parental tumor grow at the same rate in nude mice. (For further details see References 12 and 13.)

tumors were retransplanted into normal mice to determine whether these tumors had become heritable progressor variants. Table I summarizes the results. Variants that escaped immune defenses of the normal host have become resistant to activated Mϕ and tumor-specific T cells yet retained their sensitivity to NK cells. This suggests that NK cells do not play a role in the resistance of the normal host against these cancer cells. In contrast, the resistance of the host-selected progressor variants to activated Mϕ and cytolytic T cells suggests that these two types of leukocytes have tumoricidal activity in vivo.

Variants selected in vitro by either activated Mϕ or cytolytic T cells showed that loss of the cytolytic T cell-recognized antigen and resistance to Mϕ were acquired independently of each other (12, 13). However, resistance to Mϕ did not occur in a T cell-deficient nude mouse, and it is obvious that these two cell types must act together in vivo, for example, by the secretion of Mϕ activating factors from the tumor-specific T cells. Variants selected in vitro for Mϕ resistance showed a significantly increased growth potential (Figure 4). However, retention of the cyto-

lytic T cell-recognized antigen seemed to be sufficient to guarantee rejection, even though the time interval required for rejection of these Mϕ-resistant variants was significantly prolonged. Thus, sensitivity to activated Mϕ was not the sole determinant for a regressor or progressor phenotype. Nevertheless, tumoricidal Mϕ certainly seem to contribute to the defense of the syngeneic host system against the growth of cancer cells. Furthermore, the importance of tumoricidal Mϕ may become more apparent with tumors that are highly sensitive to activated macrophages yet express T cell-recognized tumor antigens that are weaker than those on the tumor 1591.

The high frequency of heritable progressor variants in UV-irradiated mice (11) (Table I) is surprising but may occur because UV irradiation leaves a partial immunity behind (1). This partially compromised immune system may favor the outgrowth of tumor variants. The mechanism might be analogous to the known medical problem of selection for drug-resistant microbial variants in patients that are treated with an insufficient dose of antibiotics. Thus, an incomplete or partially effective immunotherapy may lead similarly to the rapid outgrowth of poorly immunogenic variants.

Immune Surveillance

In 1909 Paul Ehrlich (30) suggested that, in humans without immunologic defenses, the incidence of cancer might be exceedingly high. This hypothesis postulating a resistance of the normal host against cancer—often referred to as "immunosurveillance"—only received major attention after having been restated and redefined by Thomas (31) and Burnet (32). Extensive experimentation and clinical studies have, however, indicated that, with a few exceptions, such immune surveillance does not operate effectively (33); immune surveillance may eliminate some virally induced cancers (3, 34, 35), but virally induced malignancies probably account for only a small portion of human cancers (36). Individuals with congenital immune deficiency syndrome develop only an increased incidence of cancer in the organ system that is affected by the genetic abnormality (37); such individuals, however, are usually highly susceptible to infectious diseases. Similarly, nude mice, which are congenitally athymic and, thus, do not have functional T cell immunity, are highly susceptible to infectious diseases but do not show an increased incidence or shortened latency period of chemically induced cancer (38); only the incidence of lymphoreticular tumors is increased (39). Thus, the immune system provides effective immune surveillance and a selective advantage against infectious disease, but apparently not against the induction of cancers caused by chemical or physical carcinogens.

It is important to note that the absence of immunosurveillance of cancers

induced or caused by chemical or physical carcinogens does not imply that these tumors are not antigenic. As noted earlier, UV and certain chemically induced tumors can be so highly antigenic that they are rejected by normal syngeneic hosts without prior immunization. Such tumors apparently retain their antigenicity because the immune system of the host was suppressed either by the carcinogen itself (1) or as a result of advanced age of the host (28, 29); i.e., in the absence of immunity, poorly immunogenic variants are not favored in selection, but partial immunity after UV radiation may be responsible for outgrowth of progressor variants (11).

Effects of Cancer Cells or Carcinogens on the Immune System

Immune Suppression by Tumor Cells

It has been well documented in a number of different tumor models that the tumor-bearing host has suppressed immune functions (40, 41; for review, see Reference 42). We will discuss here some important problems of tumor-induced immune suppression in the UV-induced tumor model. The advantages of the model relate to the specificity and strength of suppression in tumor-bearing mice and to the possible relationship of carcinogen-induced immune suppression to that induced by tumor burden.

To our surprise, we discovered that the immune suppression in mice bearing UV-induced progressor tumors was so strong as to permit the outgrowth of highly immunogenic UV-induced regressor tumors that are rejected regularly by the normal host (9). However, allogeneic tumors were still rejected by these hosts. Similarly, we found that the proliferative response to allogeneic tumors was as vigorous as that evinced by normal spleen cells, and that there was no suppression of the cytolytic response to alloantigen nor a suppression of the IgM antibody response to sheep red blood cells. The suppression could be transferred into other mice by theta-bearing spleen cells from mice bearing progressor tumors, but not from nude mice bearing these tumors. Other experiments showed that the suppression was caused by several different progressor tumors and affected the rejection of several different regressor tumors regardless of whether they had been induced by UV irradiation. The broad cross-reactivity of the described T cell-mediated suppression is in striking contrast to cytolytic and helper T cell-mediated immunity which we have found to be exceedingly tumor-specific for individual tumors despite careful testing for cross-reactivity. Interestingly, this striking discrepancy in specificity between the helper or cytolytic T cells on one hand and

the suppressor T cells on the other is consistent with the clear demonstration of T cell receptor rearrangements in helper and cytolytic T cells and their absence in suppressor T cell lines (43–45).

At present, we do not understand the mechanisms by which the progressor tumors induce immune suppression. The progressor tumor has to reach a critical size before the host's ability to reject highly immunogenic regressor cells is totally abrogated. However, even the burden of a large regressor tumor fails to induce immune suppression de novo (Mullen, C., unpublished results), and it appears, therefore, that other distinguishing characteristics of the progressor tumor must explain the potential of these tumors to induce immune suppression. One distinguishing feature is the capability of the progressor variants but not the parental regressor to induce NK activity (10). It has recently been shown that NK cells can eliminate dendritic cells that have been exposed to antigen (46). Furthermore, the induction of such NK activity can result in the abrogation of an immune response to antigens (47). It is conceivable that the induction of NK cells by the progressor tumor leads to elimination of dendritic cells in vivo and to the failure of the tumor antigen to be presented properly to tumor-specific T cells. This may in turn lead to the induction of suppressor T cells. At present, it is not known why the loss of a strong tumor-specific antigen makes the variant capable of inducing NK activity. However, we have shown that the tumor-specific rejection antigen that is lost by the progressor variant is an altered class I major histocompatibility complex (MHC) molecule (48). Other investigators have postulated an inverse relationship between the level of MHC class I antigen expression and the induction of or sensitivity to NK cells (49). Thus, the loss of the MHC class I-like antigen may be directly related to the induction of NK cells by the progressor tumors. In any event, evidence is accumulating that NK cells may have important immunoregulatory functions, although it remains to be shown whether the induction of NK cells by progressor tumors is causally related to the induction of immune suppression to tumor antigens.

Immune Suppression by Carcinogen

Many carcinogens are immunosuppressive (50); one of the most striking examples is UV light (1, 51, 52). Even rather small doses of UV irradiation of mice cause an immune suppression that makes mice susceptible to challenge with highly immunogenic UV-induced regressor tumors (Figure 3, Table I). The possible mechanisms involved in the induction of this suppression are still unclear although they have been discussed in detail in previous reports (1, 51, 52). Interestingly, UV radiation causes

multiple manifestations of suppression that are also found in tumor-bearing animals (9): it (*a*) permits the outgrowth of UV-induced regressor tumors, (*b*) is not specific for individual tumors, (*c*) does not affect allogeneic and other humoral responses, (*d*) is transferable with Ly-2$^-$ T lymphocytes, and (*e*) suppresses tumor rejection mounted by naive unimmunized mice but not protection conferred by lymphoid cells from mice previously immunized with a particular tumor. Together these findings suggest that carcinogens and progressively growing tumors may bring about suppression of the immune system in a similar way (9). Carcinogen treatment will lead to a large number of cellular alterations even when cancer is not induced. Whether or not the target cells become actually transformed, the carcinogen treatment may, therefore, lead to the expression of similar genes whose products induce suppression of the immune system in a similar way. Consistent with this idea are experiments indicating that transplantation of UV-irradiated skin can suppress the rejection of tumors (53, 54). The general relevance of our findings (9) to tumor immunology is indicated by the fact that tumors other than those caused by UV can induce and are susceptible to the suppression described here, and that carcinogens in general cause immunosuppression by altering the phenotype of a large number of cells.

At present, there still is a major problem of defining the nature and mechanism of action of suppressor T cells. However, one cannot disregard the fact that the suppression induced by a tumor burden or the carcinogen is very profound. It has been proposed that highly immunogenic regressor tumor sublines isolated from a progressively growing tumor may be useful

Table II. Characteristics of Unique Tumor-specific Antigens

1. Specific for a particular tumor even when compared to other tumors
—of the same histologic type
—induced in the same organ system
—by the same carcinogen
—in the same strain of mice
2. Defined by transplantation assays (not possible in humans)
3. Found on chemically and physically induced experimental tumors
4. Seemingly endless variety
5. Not caused by clonal amplification of preexisting antigens
6. Not present on normal syngeneic cells

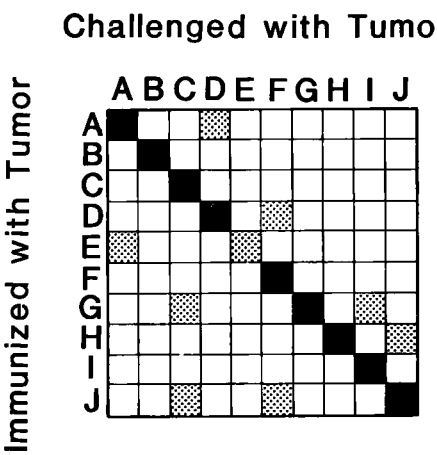


Figure 5. Demonstration of individually distinct (unique) transplantation antigens on independently methylcholanthrene-induced sarcomas derived from mice of the same inbred strain. Screening for shared antigenicity failed to yield any reproducible cross-reactions. *Solid squares*: rejection of tumor inoculum. *Dotted squares*: rejections that could not be repeated. *Empty squares*: combinations that showed no rejection. Modified from Basombrio and Prehn (78).

for active immunotherapy. Our experiments show that such highly immunogenic cells may not only form tumors in the tumor-bearing host but that such immunogenic tumor cells may even maintain existing suppression. It will certainly be important to learn how to overcome the immune suppression in the tumor-bearing host and to accomplish rejection of these extremely immunogenic UV-induced tumors; otherwise, the outlook for active immunotherapy of other cancers—many of which may be less immunogenic—is poor. Furthermore, it has been demonstrated that suppressor mechanisms stimulated by progressively growing tumors are barriers for adoptive (passive) immunotherapy (55, 56); this important subject is therefore discussed in detail elsewhere in this book.

Unique Tumor-specific Antigens

Transplantation Experiments Demonstrating Unique Tumor-specific Rejection Antigens

For decades, investigators searched for tumor antigens that were present on the cell surface of most or all cancers but absent on normal cells (and were therefore "tumor-specific"). If one excludes tumors of viral etiology—and there is very little evidence that the majority of human cancers are of viral etiology (36)—then all of the studies in mice and humans have failed to provide evidence for the existence of common, truly tumor-specific antigens despite continuing reports (57–60) that cannot be confirmed (59). This suggests that such *common* tumor-specific antigens may not exist. One unambiguous finding, however, was that each experimentally induced cancer had uniquely specific antigens (Table II and 14, 61–65). These

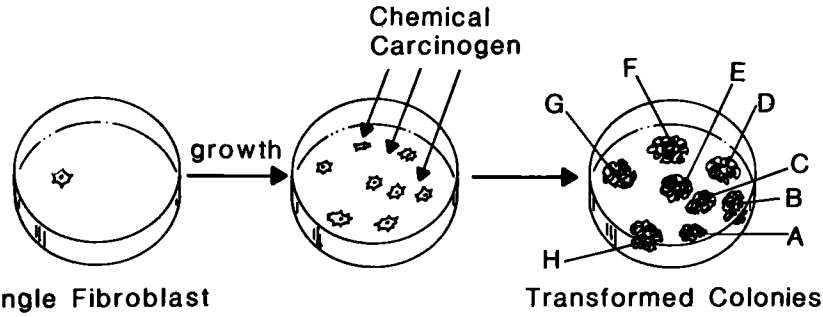


Figure 6. Scheme of experiment to test validity of the clonal amplification hypothesis. The results show that progeny of a single cell, when exposed to carcinogen in vitro, give rise to tumors that have individually specific tumor antigens. Thus, the observed diversity could not be preexistent if it was apparently induced as the result of carcinogen exposure (78, 79).

unique antigens were found to be specific for a particular tumor even when compared to other tumors of the same histologic type induced in the same organ system by the same carcinogen in the same strain of mice.

The unique tumor-specific antigens have traditionally been defined by transplantation assays. These assays involved inoculation of hosts with tumor followed by surgical excision of the growing tumor and after time rechallenge of the host with the original tumor. Normal tissue from the mouse of tumor origin or other independently derived tumors were used as controls. The remarkable antigenic polymorphism of these individually specific (or unique) antigens was first detected on chemically induced sarcomas (Figure 5; 61–65). Later work showed that other tumors (66–68) including UV light-induced tumors (22, 69) also express such unique tumor-specific antigens. It has not been possible to determine whether or not these antigens also exist on human tumors because transplantation assays cannot be used to define such antigens in humans.

Although these unique tumor-specific antigens were discovered many years ago, the molecular nature and genetic origins of these antigens have remained obscure. Immunologists have speculated that these antigens might be encoded by highly polymorphic gene families (70, 71) such as the class I genes of the MHC or the variable gene clusters encoding for immunoglobulins or T cell receptors (72–74). In contrast to idiotypes on B cell malignancies (75; for review, see Reference 77), unique tumor-specific antigens are apparently not caused by clonal amplification of preexisting antigens. The evidence for this comes from studies showing that progeny of a single precursor cell independently transformed in vitro still express individually distinct tumor-specific antigens (Figure 6; 78, 79). Thus, unique tumor-specific antigens appear to be neoantigens arising

as a result of malignant transformation, possibly because of the mutagenic action of most (and possibly all) chemical and physical carcinogens (80, 81). This notion agrees with the much older observation that unique antigens are not found on nonmalignant cells and, therefore, are truly tumor-specific (61). The fact that these antigens appear to be the only changes in cancer cells presently known to be tumor-specific has led to much speculation of whether or not these antigens might be related to or essential for the establishment of malignancy. As was suggested by Boyse many years ago (82), unique tumor-specific antigens might represent altered hormonal receptors or cell–cell recognition sites (83) needed for normal growth of differentiation; the mutation or loss of these molecules then might lead to immunogenic or nonimmunogenic tumors, respectively.

The major reason for the failure of molecular characterization of unique tumor-specific antigens has been the complete lack of probes that are uniquely specific, permanent, and transferable between laboratories. The difficulties have been additionally compounded by the fact that cell surface proteins may lose immunogenicity during purifications. We have, therefore, developed a new approach for the study of unique tumor antigens in UV-induced tumors in mice.

Syngeneic Cytolytic T Cell Lines as Probes for Unique Tumor-specific Antigens

Because the rejection of tumors used as allografts is classically T cell-mediated, we determined first whether tumor-specific cytolytic T cells

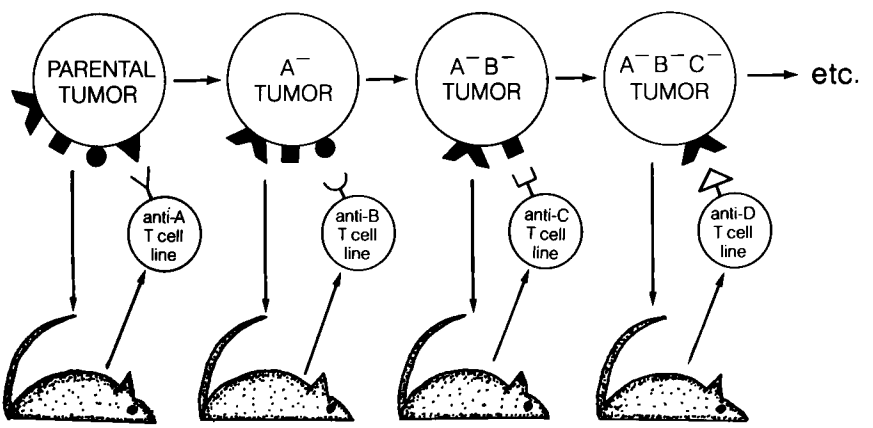
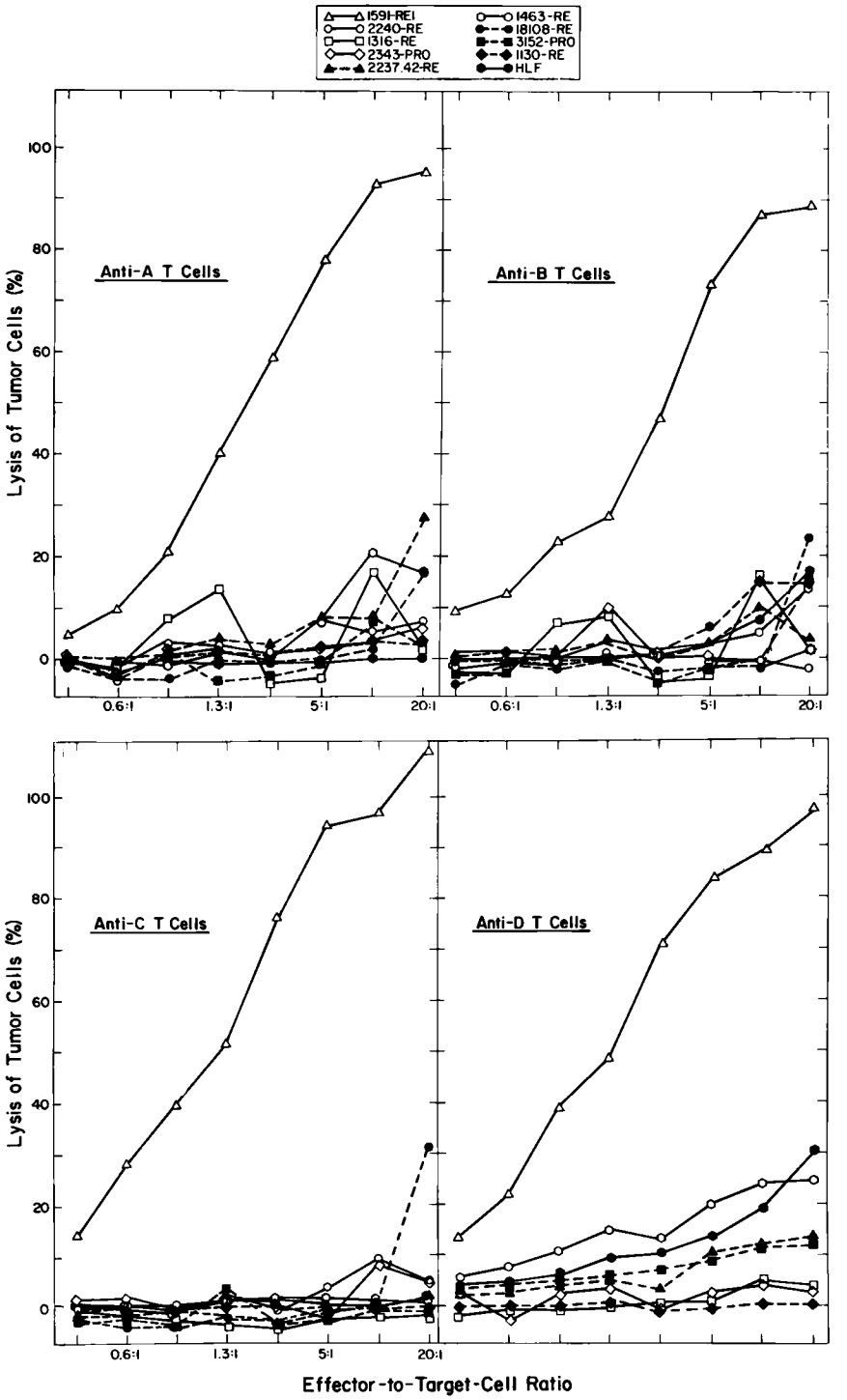


Figure 7. Schematic diagram of the sequential selection whereby the complexity of a unique tumor-specific antigen can be dissected by generating T cell lines. T cell lines are generated from mice immunized with in vitro selected tumor variants. (For further explanation see text and Reference 84.)


1591-RE1
2240-RE
1316-RE
2343-PRO
2237.42-RE
1463-RE
18108-RE
3152-PRO
1130-RE
HLF
Anti-A T Cells
Anti-B T Cells
Anti-C T Cells
Anti-D T Cells
Lysis of Tumor Cells (%)
Effector-to-Target-Cell Ratio
0.6:1
1.3:1
5:1
20:1
0
20
40
60
80
100

for syngeneic tumors could be used as permanent and specific probes to define such transplantation antigens. A syngeneic cytolytic T cell line (called anti-A because it was the first) was prepared against the UV-induced mouse fibrosarcoma 1591 (84). The A^- variants that were resistant to the anti-A T cell line grew out in vitro from this population (Figure 7). An A^- variant was then used to derive a second cytolytic T cell line ("anti-B"), which to our surprise reacted with a different antigen present on the variant as well as on the parental tumor cell. To our further surprise, we found by additional repeated cycles of selection that a single malignant cell apparently possessed a C and a D antigen as additional tumor-specific antigens (84). All of these antigens were uniquely tumor-specific (Figure 8) and it is also important to mention that all four tumor-specific antigens are always lost independently of each other (Figure 9).

The next step was to determine the importance of each of these cytolytic T lymphocyte (CTL)-defined antigens in vivo for tumor rejection. Several lines of experimental evidence supported the idea that CTL-defined, tumor-specific antigens might be important for tumor rejection (Table III). First, idiotype-specific suppression of the tumor-specific cytolytic T cell response made the host selectively susceptible to the tumors for which the response was suppressed (25, 26). Second, rare progressor variants selected by the normal hosts' immune system regularly lost a regressor tumor CTL-defined antigen (10, 11). Finally, we also took another approach to determine which of the antigens recognized by syngeneic cytolytic T cells could elicit rejection of the 1591 tumor by the normal host (13). This was done by selecting in vitro with T cell clones tumor variants with defined antigenic changes and then testing the growth of these variants in vivo.

For example using the A^+B^+ 1591 regressor tumor (1591-RE2, which does not express the C or D antigens), selection for loss of the A antigen resulted regularly in progressive growth of the A^-B^+ variant in vivo (Figure 10) whereas the A^+B^- variants did not grow progressively. Further experiments using other 1591 variants showed that the A and the C antigen had to be lost before the tumor could grow progressively in the normal host whereas the loss of the B and D antigen was not required for progressive tumor growth, indicating that loss of a single CTL-defined antigen could determine whether regressive or progressive growth occurred.

Figure 8. Unique specificity of tumor reactive T-cell lines for 1591 tumor cells. The radioactive intracellular label ^{51}Cr is released from 1591 tumor cells but not from any other UV-induced fibrosarcoma cells tested; only some control tumors tested are shown here. Reprinted from Wortzel et al. (84) with kind permission from the publishers.

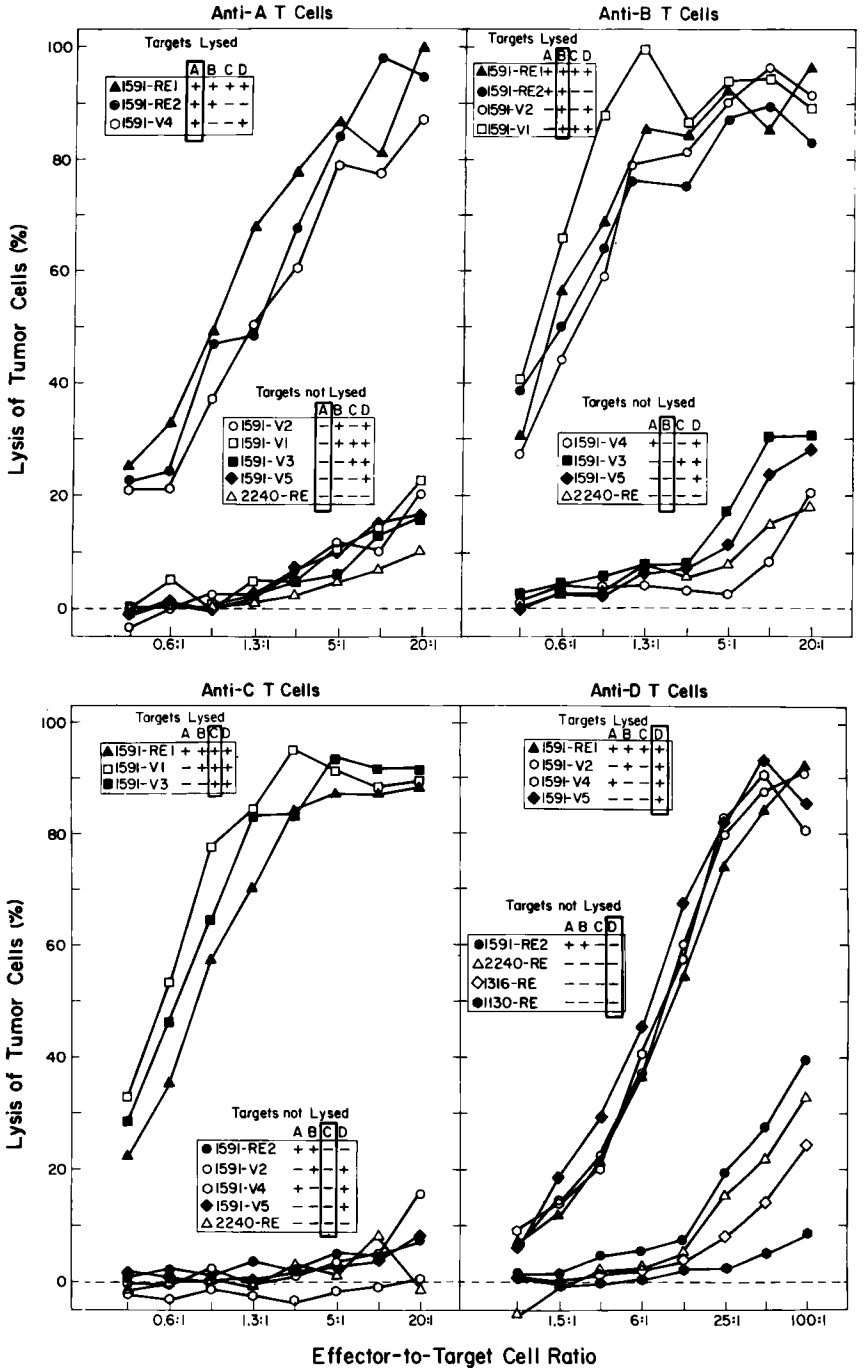

Anti-A T Cells
Anti-B T Cells
Anti-C T Cells
Anti-D T Cells
Targets Lysed
Targets not Lysed
Lysis of Tumor Cells (%)
Effector-to-Target Cell Ratio
0.6:1
1.3:1
5:1
20:1
1.5:1
6:1
25:1
100:1

Table III. Experimental Evidence for Tumor-specific Cytolytic T Cells Recognizing Transplantation Antigens

1. Idiotype-specific suppression of CTL responses leading to selective loss of tumor resistance (25)
2. Loss of CTL-defined tumor-specific antigens from progressor variants (10)
3. In vitro selection of in vivo progressor variants with regressor antigen-specific CTL clones (13)

Syngeneic Monoclonal Antibodies as Probes for Unique Tumor-specific Antigens

Although cytolytic T cell clones have proven to be excellent probes for defining essential tumor-specific transplantation antigens, these probes cannot be used directly for biochemical analysis. Thus, our next approach was to generate monoclonal antibodies that would have the same reactivity pattern and select for the same variants as A antigen-specific CTL clones (48). We preferred this approach over selecting hybridomas that specifically could block anti-A CTL clones because the latter approach would not detect some A antigen-specific hybridomas. Because the major immune response to transplantation antigens of tumors is dominantly T cell- rather than B cell-mediated (85, 86), difficulties in making such specific hybridomas must be expected. In our hands, hyperimmunization or isologous immunization have only yielded cross-reactive hybridomas. We have had success with immunization of syngeneic mice once with viable tumor cells followed 4 weeks later by a single intravenous boost 3 days before fusions. The overall frequency of specifically reacting hybridomas is still exceedingly rare, i.e., about 1 in 2,000–3,000 growing hybridomas. The specific hybridoma CP28 selected for variants resistant to anti-A T cells; although the reciprocal was also true, no other marker was affected. Monoclonal antibody CP28 did not react with any in vivo selected progressor variants nor with normal cells (Figure 11). Finally, Figure 12 shows that this monoclonal antibody does not react

Figure 9. Multiplicity and independence of 1591-specific antigens as demonstrated by cytolytic T cells and immunoselected variants. The radioactive label ^{51}Cr is only released from tumor cells expressing the relevant unique tumor-specific antigen for which the T-cell is specific. Reprinted from Wortzel et al. (85), with kind permission from the publisher.

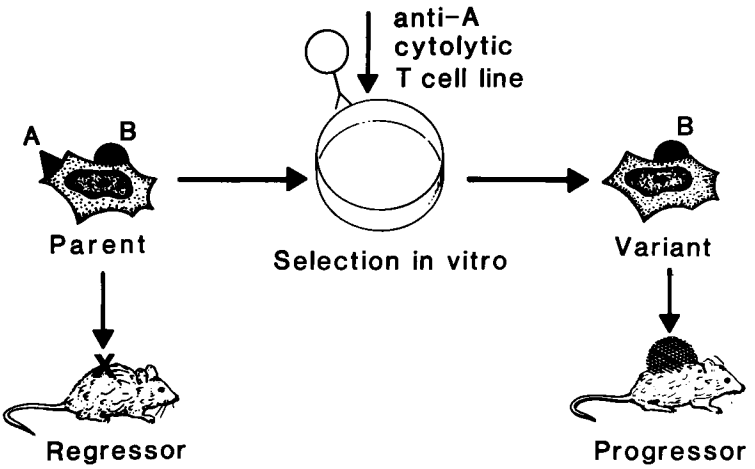


Figure 10. Scheme of experiment to test the relevance of a CTL-defined unique tumor-specific antigen for tumor rejection in vivo. Loss of the A antigen leads to variants showing progressive tumor growth in normal mice while the reciprocal variants selected for loss of the B antigen are still rejected by the normal host. (For further details see Reference 13).

with 37 other syngeneic tumors tested. A second hybridoma, CP3F4 (not shown) produced antibody of similar specificity.

Although these monoclonal antibodies failed to react with syngeneic normal or other malignant cells, these antibodies did react with public specificities of class I molecules of several other strains of mice (48). To prove that the monoclonals indeed cross-reacted with allogenic class I molecules, L cells transfected with class I genes were tested. Our results showed that the monoclonals reacted with the transfectants in perfect agreement with genetic mapping studies using microcytotoxicity tests (48). It was, however, apparent from testing the gene transfectants that the molecule on 1591 is not a bona fide allogeneic class I molecule (48). To identify further the molecular nature of this unique tumor-specific antigen, we immunoprecipitated radiolabeled lysates from 1591 cells. The precipitated molecules showed the typical two-dimensional gel pattern of a class I MHC molecule (48). These molecules were totally absent from the variant cells selected for loss of the antigen recognized by these monoclonals. Preliminary results suggest that the altered class I gene products that are recognized by CP28 or CP3F4 monoclonal antibodies are also recognized by the syngeneic anti-A CTL and that these altered class I gene products indeed represent unique tumor-specific rejection antigens of the tumor (87).

At present, we do not know whether all the other unique tumor-specific

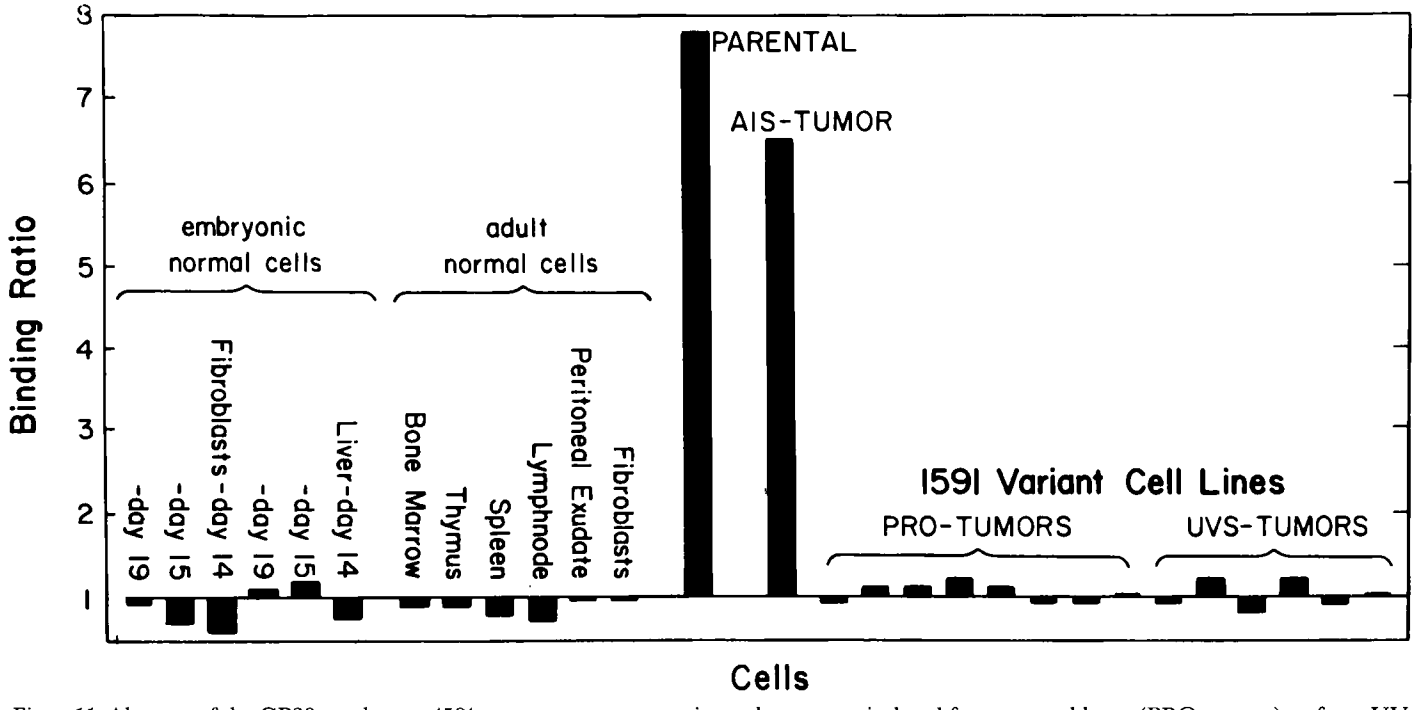


Figure 11. Absence of the CP28 marker on 1591 progressor tumor variants that were reisolated from normal hosts (PRO tumors) or from UV-irradiated hosts (UVS tumors). The marker is not lost from tumors that grow progressively in idiotypically suppressed mice (AIS tumors) incapable of generating a tumor-specific CTL response. Antibody CP28 does not bind to the various normal syngeneic adult and embryonic cells tested. Cells were incubated first with CP28 antibody and then with fluorescein-coupled goat anti-mouse immunoglobulin antibodies. Cells were analyzed with the fluorescence-activated cell sorter (FACS IV) and the amount of fluorescence was recorded in logarithmic channel numbers. Although autofluorescence can vary considerably among different cell lines, we determined the binding ratio, i.e., the ratio of the amount of fluorescence after staining with both antibodies divided by the amount of fluoresence after staining with the second antibody alone, for each cell line. Reprinted from Philipps et al. (48) with kind permission from the publisher.

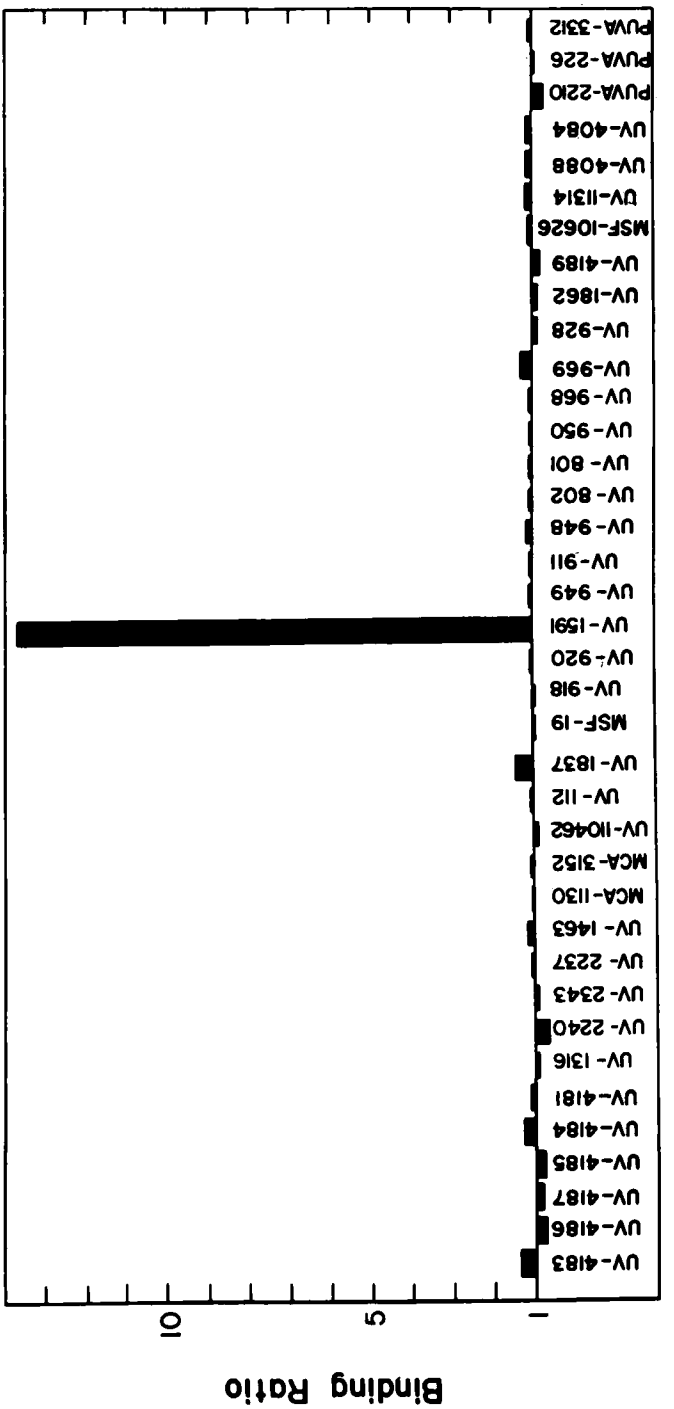


Figure 12. Unique tumor-specificity of the syngeneic monoclonal antibody CP28 when tested on a battery of 37 other syngeneic fibrosarcoma lines. The binding ratios were determined as described in the legend of Figure 11. Reprinted from Philipps et al. (48) with kind permission from the publisher.

antigens that we have shown to exist on the 1591 tumor belong in a family of related molecules or whether they are of diverse origin. In this regard, it is interesting that the methylcholanthrene-induced fibrosarcoma Meth A has a unique tumor-specific antigen whose expression is closely linked to the immunoglobulin gene cluster (88, 89). The Meth A fibrosarcoma appears to be the only other tumor whose unique tumor-specific transplantation antigen is comparably well described. Both Ig and MHC class I molecules are related to cellular recognition structures (90), and abnormalities in such molecules may lead to abnormal growth and differentiation which is possibly related to malignant behavior per se. At present we do not know, however, why the novel MHC class I molecules are synthesized. Certainly, germline mutations are not likely to account for the strong antigenicity observed with UV-induced cancers in that more than 90% of such cancers are regressor tumors that are rejected when transplanted into normal syngeneic hosts (1). Finally, 1591 clearly originated from a genetically normal C3H/HeN mouse by numerous criteria such as isozyme tests (48). Furthermore, other experimentally induced cancers have been shown to be antigenic in the autochthonous host (14). Thus, it is much more likely that the novel class I genes originated because of mutagenic action of the carcinogen during tumor induction or to the increased genetic instability that is associated with tumor progression. We know that the novel class I products on the 1591 tumor do not appear as a result of derepression of preexisting complete class I genes because we find clear evidence of genetic rearrangement of class I MHC genes by Southern blot analysis (87). Thus, either somatic recombination or gene conversion appear to have occurred. Such mechanisms of altering MHC class I genes could undoubtedly generate virtually endless numbers of unique tumor-specific antigens.

At present, we do not know whether unique tumor-specific antigens are frequently or rarely like MHC class I molecules. However, it is important to mention that numerous earlier experiments have suggested the existence of altered MHC molecules on other tumors (91–93) although the relationship of this altered MHC expression to the unique, i.e., individually specific tumor antigens as described here (48), has remained obscure in the previous work. It is still contradictory (94, 95) whether quantitative changes of MHC class I expression commonly affect the degree of malignant behavior of tumor cells. For certain tumors (96, 97), however, loss of MHC class I antigens has clearly been shown to affect immune responses supposedly because of loss of a restriction element required for T cell recognition (for review, see Reference 98). Certain tumor cells can also be made immunogenic by transfecting them with

normal MHC class I genes (99–101) supposedly by restoring a lost MHC restriction element. It is unclear, however, whether or not abnormal class I antigens may cause a tumor cell to become rejected. In any event, the nature of the supposedly MHC-restricted, tumor-specific antigen remained elusive in these studies (99–101).

Pecking Order among Tumor-specific Antigens, Antigenic Variation and Tumor Progression

We have shown above that a single cancer cell can express multiple unique tumor-specific antigens (84). These tumor antigens are recognized by syngeneic CTL clones, and it would definitely be to the host's advantage if all unique antigens were recognized at one time. Such recognition would greatly decrease the likelihood of escape of antigen loss variants from the host's immunity. Unfortunately, the host clearly fails to respond to all of these antigens simultaneously (102). For example, all variants that express the A antigen, e.g., $A^+B^+C^-D^+$, $A^+B^+C^-D^-$, $A^+B^-C^+D$, etc., elicit a specific CTL response limited to or predominantly directed to the A antigen. Even repeated immunizations with these tumor cells only elicit a response to the A antigen. Only when the A antigen is lost from the tumor is the next dominant antigen recognized. This pecking order of the host response to multiple unique tumor-specific antigens not only causes sequential antigenic variation in a tumor population, but also leads to tumor progression, because the antigenic loss variants also have an increased malignant growth behavior, e.g., only 10^4 cells of the $A^-B^-C^-D^-$ 1591 variants kill mice in about 3 weeks whereas 10^6 $A^-B^+C^+D^+$ kill most mice in 5–6 weeks (Van Waes, C. unpublished results). Some of these variants are also metastatic and it is not known whether any other tumor-specific antigens still remain on these highly immunoselected tumor variants $A^-B^-C^-D^-$. Together it appears that the parental highly immunogenic tumor, by inducing a specific cytotoxic response, favors the outgrowth of less immunogenic variants. Obviously, an aim for the future is to prevent the outgrowth of such immunoselected variants. One approach might be to immunize the host during an early stage of cancer with more malignant progressor variants derived in vitro before they actually arise in vivo. This might be feasible because progressor variants very closely simulating those arising in the intact host can be generated in culture under defined conditions using specific monoclonal antibody or CTL lines (13).

Conclusion

Ultraviolet light-induced murine tumors are strongly antigenic and are usually rejected by normal syngeneic animals. In rare instances, however, the combination of selection by the immune system and the genetic instability of the cancer cells results in outgrowth of variants that are antigenically altered and grew progressively in normal recipients. An analysis of these escape variants provides a way for determining how the normal host may respond to a tumor and how tumors may respond to the immune system.

We find that CTL and activated Mϕ are tumoricidal effector cells involved in the rejection in the UV-induced tumors, whereas NK cells are not. In fact, variants that grew progressively induce NK cells more effectively than the parental regressor tumor. This paradoxical finding for NK cells may depend on the fact that NK cells adversely affect T cell-mediated immunity to the tumor.

The first unique tumor-specific antigen that we identified on a parental or regressor UV tumor was a novel class I MHC molecule. At present, we do not know whether other unique tumor-specific antigens are frequently or rarely like class I molecules or whether these unique antigens are related to the malignant behaviour of the tumor cells. A single malignant cell can carry multiple independently expressed unique tumor-specific antigens; however, a "hierarchy" in the immune response prevents generation of specific immunity except to the first or dominant tumor antigen. This characteristic of the immune response undoubtedly accounts for the phenomenon of escape by variants that lose the dominant antigen. But as we have also shown, specific immunity can be raised against the other antigens suggesting ways for immunizing individuals against all the antigens present on any tumor. Such immunization should prevent outgrowth of variants.

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Generation and Suppression of the Immune Response to Immunogenic Tumors

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Introduction

The existence of tumor-specific transplantation antigens was demonstrated repeatedly in the 1950s and 1960s by showing that mice can be immunized against syngeneic or autochthonous chemically induced tumors (1–5). An immunogenic tumor, then, is one against which a syngeneic host can be immunized, either by removing the tumor by surgery or ligation after allowing it to grow for a while, or by injection of heavily irradiated cells of the tumor, with or without adjuvants. Evidence that successful immunization has been achieved is indicated by the ability of the host to resist the growth of a challenge implant of cells of the tumor used for immunization, but not of other tumors. Indeed, the immunity acquired in response to immunization is, in most cases, surprisingly specific, in that it is not expressed against other syngeneic tumors induced by the same chemical in the same tissue of the same strain of mice. It was not long after the demonstration of tumor immunogenicity that it was shown that antitumor immunity, like antiallograft immunity, is cell-mediated, in that it can be passively transferred to syngeneic recipients with lymphoid cells, but not with serum. More recent experimentation in vivo and in vitro (5) leaves no doubt that the lymphoid cells that passively transfer immunity are T cells.

Needless to say, the demonstration that certain tumors can possess tumor-specific transplantation antigens posed the problem of explaining why such tumors arise in their autochthonous hosts in the first place, and why they grow progressively without interruption when transplanted to syngeneic hosts, in the second. Numerous attempts have been made

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to explain this problem, including one based on the idea that solubilized tumor antigens or antigen–antibody complexes "block" the antitumor function of tumor-sensitized T cells (6, 7). It also has been suggested (8, 9) that tumors secrete anti-inflammatory factors that inhibit the migration of host effector T cells from blood to tumor. However, it is difficult to totally reconcile these explanations with recent demonstrations (10) that relatively large established tumors can be made to undergo complete regression by infusing the tumor-bearing mice with enough sensitized T cells from immunized donors (see below). These demonstrations leave little doubt that the reason that immunogenic tumors are not destroyed is because their hosts fail to generate a sufficient number of effector T cells. Indeed, most explanations of the escape of immunogenic tumors from immunity have taken second place to more recent explanations based on the negative immunoregulatory function of suppressor T cells. According to these explanations, the antitumor immune response is downregulated by tumor-induced suppressor T cells before enough effector T cells are generated to cause tumor regression.

There are two major models of T cell-mediated suppression of antitumor immunity. One is based mainly on the ability of T cells from tumor bearers to suppress the expression of a delayed sensitivity reaction to tumor antigens in immunized recipients (11). The other is based on the ability of T cells from tumor bearers to suppress the expression of adoptive immunity against an established tumor in immunodepressed recipients (5). Because the second mentioned model is understood in more detail, and because it is the one that is being employed in this laboratory to analyze the immune response to several immunogenic tumors, it will be given the most attention in this article.

Presence of Suppressor T Cells Is Revealed by Their Ability to Suppress the Expression of Adoptive Immunity against an Established Tumor

Although there are numerous published demonstrations that passively transferred, tumor-sensitized T cells can inhibit the growth of an implant of tumor cells, there appears to have been only one demonstration (12), until recently, that the same sensitized T cells can cause the regression of a tumor once it is established and growing progressively (13). Apparently, therefore, something happens in the host, between the time of implanting tumor cells and the emergence of a palpable tumor, that prevents intravenously infused tumor-sensitized T cells from expressing their antitumor function. It was postulated that this obstacle to adoptive immunotherapy is the acquisition by the host of tumor-induced suppressor T cells. This postulate was tested by determining whether tumor regres-

sion would occur if the recipient of tumor-sensitized T cells were made incapable of generating suppressor T cells. This involved attempts to immunize against an established tumor adoptively in recipients that had been immunodepressed to prevent them from making a T cell response.

For these experiments donor mice were immunized by injecting them with an admixture of living tumor cells and the adjuvant, *Corynebacterium parvum* (14). Donor spleen cells were harvested 30 days after giving the admixture, when the donors are known to possess a state of immunologic memory (15). The recipients were permanently immunodepressed by thymectomy and lethal gamma irradiation followed by bone marrow reconstitution (TXB mice), or temporarily immunodepressed by sublethal (500 rads) gamma irradiation alone, or by injection of a single 100 mg/kg dose of cyclophosphamide.

It was shown with the Meth A fibrosarcoma, for example, that, whereas intravenous infusion of spleen cells from immunized donors failed to cause regression of a 4-day tumor in immunocompetent recipients, the same number of immune spleen cells caused complete regression of the same sized tumor growing in TXB (16), irradiated (17), or cyclophosphamide-treated (18) recipients (Figure 1). The key additional finding in all cases was that tumor regression could be inhibited by infusing the adoptively immunized recipients with splenic T cells from donors bearing an established tumor, but not with spleen cells from normal donors. Therefore,

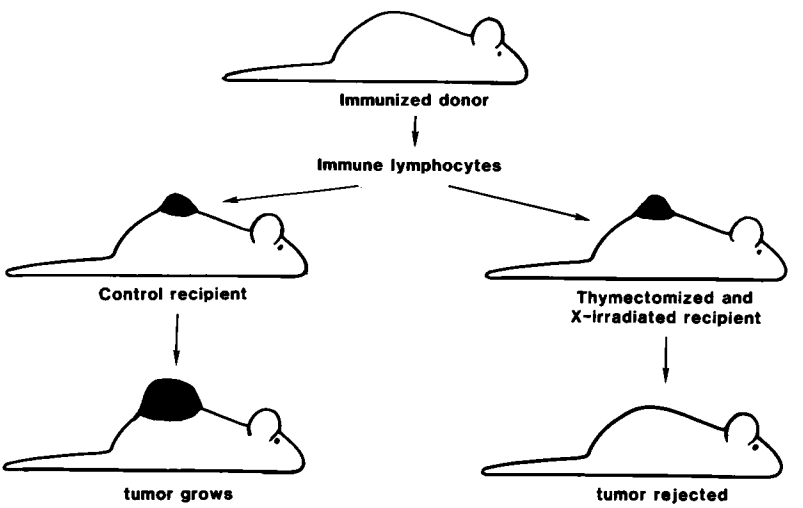

Figure 1. It is now well established that, in order for T cells from immunized donor mice to cause the regression of an already established tumor in recipient mice, the recipients need to be immunodepressed.

tumor-bearing, immunocompetent mice acquire tumor-induced lymphocytes capable of suppressing the ability of infused immune lymphocytes to cause tumor regression.

This passive transfer assay of tumor-induced immunosuppression is depicted diagrammatically in Figure 2. It was used to show that the immune memory cells in immunized mice that passively transfer immunity are Ly-1$^+$2$^-$ T cells (19), and that the suppressor cells from tumor bearers that suppress the expression of passively transferred immunity are Ly-1$^+$2$^-$ T cells (20, 21). The assay was also used to show that immune T cells and suppressor T cells are specific for the tumor that evokes their generation (22), and that the extent of suppression is determined by the ratio of suppressor to effector T cells infused into the TXB tumor-bearing test recipients (23). More recent experiments have shown that the suppressor T cells besides having the Ly-1$^+$2$^-$ phenotype, also display the L3T4a surface antigen (DiGiacomo and North, to be published).

Tumor-induced Ly-1$^+$2$^-$ Suppressor T Cells Are Generated during the Loss of an Acquired Population of Ly-1$^-$2$^+$ Effector T Cells

It is one thing to demonstrate with a given assay that suppressor T cells are generated in response to the growth of an immunogenic tumor, but it is another thing to explain how the suppressor cells allow immuno-

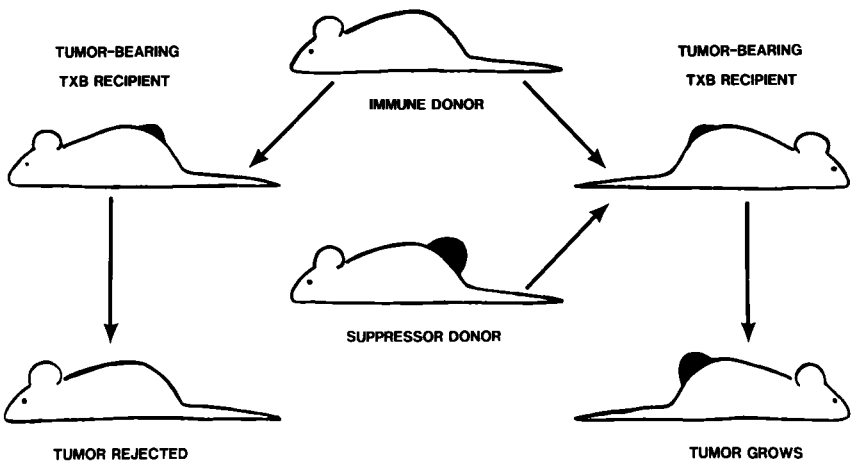

Figure 2. Diagrammatic representation of the physiologic in vivo assay employed to measure the production of suppressor T cells in tumor-bearing mice. The assay is based on the ability of suppressor T cells from tumor bearers to suppress, on passive transfer, the ability of passively transferred T cells from immunized donors to cause tumor regression in an immunodepressed (TXB) recipient.

genic tumors to avoid destruction by an antitumor immune response. Indications that suppressor T cells function to down-regulate an already ongoing antitumor immune response are given in publications dealing with concomitant antitumor immunity (24). Concomitant immunity refers to a paradoxical state of immunity acquired in response to progressive tumor growth that enables a tumor-bearing host to specifically inhibit the growth of an implant of cells of the same tumor. A key characteristic of concomitant immunity is that it can undergo rapid decay after the primary tumor grows beyond a certain critical size. This suggests the possibility that concomitant immunity decays, because it is actively down-regulated by suppressor T cells. If so, progressive growth of an immunogenic tumor should first evoke the generation of effector T cells, and then result in the progressive loss of these effector T cells in concert with the progressive production of suppressor T cells. This possibility was investigated in this laboratory by employing passive transfer assays capable of measuring changes in the acquisition of effector and suppressor T cells against time of tumor growth.

Effector T cell production was followed by determining whether the spleens of tumor-bearing donor mice acquire and then lose T cells capable, on intravenous infusion, of causing regression of a relatively small tumor in gamma irradiated (500 rads) recipient mice. It was found that mice bearing the Meth A fibrosarcoma (20) or P815 mastocytoma (21) acquire T cells capable of causing regression of a recipient tumor. These T cells were first detected on day 6 of tumor growth, reached peak number on about day 9, and were then progressively lost as the tumors increased in size. In mice bearing the Meth A fibrosarcoma, the acquisition and loss of T cells capable of passively transferring immunity were accompanied by the generation and loss of concomitant immunity to growth of a tumor implant (20). In the case of the P815 mastocytoma, the generation and loss of T cells capable of passively transferring immunity corresponded to the generation and loss of T cells cytolytic for P815 cells in vitro (21). This is in keeping with the additional finding with both tumors that T cells capable of passively transferring immunity were predominantly of the cytolytic Ly-2$^+$ phenotype (20, 21).

The kinetics of suppressor T cell production against time of growth of the same tumors was investigated by employing the standard suppressor assay described above. It was found that T cells capable, on passive transfer, of suppressing the expression of adoptive immunity against a tumor in TXB recipients were first generated on about day 9 of tumor growth and increased progressively in number thereafter (20, 21). Thus, suppressor T cells were progressively acquired as effector T cells were progressively

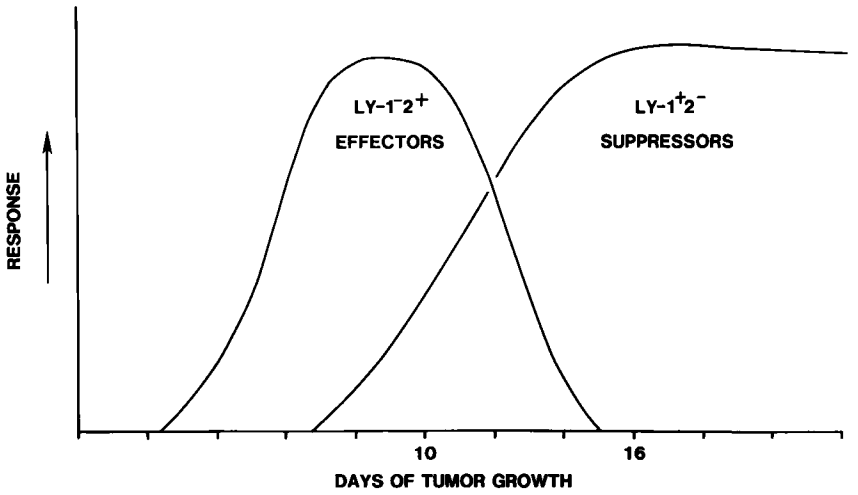

Figure 3. Diagrammatic representation of the inadequate immune response to immunogenic tumors. According to the results obtained with several tumors, the host generates effector T cells progressively until about day 9 of tumor growth, when the tumor is about 8 mm in diameter. After day 9, however, effector T cells are progressively lost, and this is associated with the progressive production of tumor-specific suppressor T cells.

lost. The kinetics of effector T cell production and suppressor T cell production during progressive growth of the Meth A fibrosarcoma and P815 mastocytoma are represented diagrammatically in Figure 3. It can be seen that the curve for suppressor T cell production is the reciprocal of the curve for effector T cell production. This means that tumor-induced suppression is not a sudden all-or-none event, but involves a progressive increase in the ratio of suppressor to effector T cells, until suppressors become dominant.

Discussion

The purpose for postulating the existence for suppressor T cells in tumor-bearing mice is to explain how immunogenic tumors avoid destruction by the antitumor immune response of their immunocompetent hosts. Two types of suppressor T cells have been shown to be generated in response to tumor growth: (*a*) suppressor cells that are capable, on passive transfer, of suppressing a delayed-type hypersensitivity (DTH) reaction in tumor-immune recipients and (*b*) suppressor cells that are capable of suppressing the expression of adoptive immunity against an established tumor in TXB recipients, as described in this article. Tumor-induced suppressors of DTH have been studied mainly in A/J mice bearing the

S1509a sarcoma (11), although very similar findings have recently been obtained with the BALB/c Meth A fibrosarcoma (25, 26). Suppressors of DTH are generated before effector T cells are lost, according to results presented here (20, 21), in that they are produced within 48 hours of injecting 10^6 tumor cells subcutaneously, reach peak production on about day 6 of tumor growth, and then progressively disappear from the spleen (25). These suppressors display the Ly-1^-2^+ phenotype, as do the T cells that suppress DTH reactions in other models of suppression (11, 25, 26).

It may well be asked, therefore, whether there is any connection between the Ly-1^-2^+ suppressors of DTH and the subsequently produced Ly-1^+2^- suppressors that inhibit the expression of adoptive immunity against an established tumor in TXB recipients. This question has little meaning, however, without a knowledge of whether both types of suppressors are generated in response to the same tumor. In fact, results recently obtained in this laboratory (DiGiacomo and North, to be published) show that both types of suppressors are generated in response to the Meth A fibrosarcoma under study in this laboratory. Their kinetics of generation are compared diagrammatically in Figure 4. Whether a functional connection exists between these two types of suppressor T cells is currently under investigation. It can be stated now, however, that the results of recent

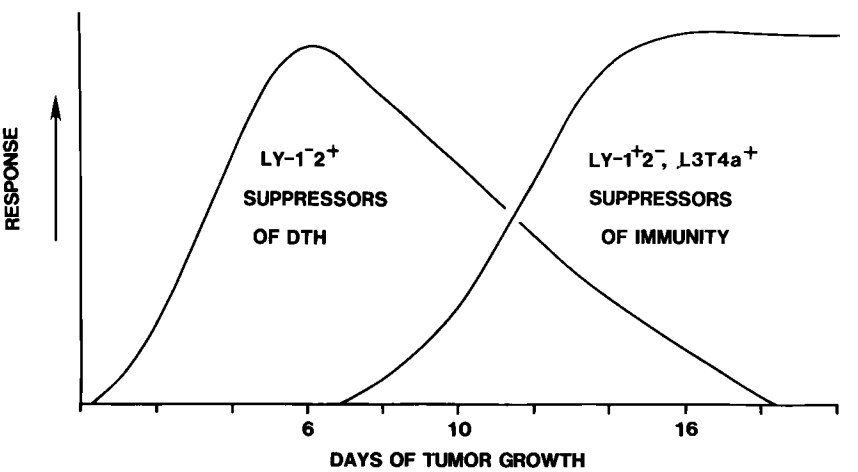

Figure 4. Diagrammatic representation of the results of a recent investigation (to be published) of the production of Ly-1^-2^+ T cells that suppress the expression of a DTH reaction in immunized recipients versus the production of Ly-1^+2^- T cells that suppress the expression of adoptive immunity. Suppressors of DTH are generated before effector T cells, whereas suppressors of adoptive immunity are generated while effector T cells are being lost.

experiments show (DiGiacomo and North, to be published) that the T cell suppressors of DTH fail to suppress the expression of adoptive immunity in T cell-deficient recipients. Conversely, the T cell suppressors of adoptive immunity fail to suppress the expression of DTH. It is important to point out, moreover, that others have shown with the Meth A fibrosarcoma (25) that Ly-1$^-$2$^+$ suppressors that inhibit DTH in tumor-immunized recipients also fail to inhibit immunity against the growth of a tumor implant in the same recipients. Therefore, the evidence that Ly-1$^-$2$^+$ suppressors of DTH function to suppress a mechanism of immunity capable of rejecting an implant of tumor cells, not to mention an established tumor, is not very convincing. It should also be pointed out that tumor-induced Ly-1$^-$2$^+$ T cells that suppress DTH, like other Ly-1$^-$2$^+$ DTH suppressors, inhibit the expression, rather than the induction of a state of DTH. In fact, there is recent convincing evidence (27) that the T cells that suppress the induction, as opposed to the expression, of DTH to peptide antigens bear the Ly-1$^+$2$^-$, L3T4a$^+$ helper phenotype.

In this connection, experiments performed to investigate the meaning of T cell-mediated suppression of adoptive antitumor immunity in TXB recipients, as described in this laboratory, revealed that the suppression of adoptive immunity against an established tumor by intravenously infused, Ly-1$^+$2$^-$, L3T4a$^+$ suppressor T cells is associated with a failure of donor memory T cells to give rise to effector T cells in the TXB test recipient (19). Thus, when cytolytic T cell production was observed in the draining lymph nodes of tumor-bearing recipients infused intravenously with immune T cells alone, or immune T cells plus suppressor T cells, it was found that only the former recipients generated appreciable numbers of cytolytic T cells, and did so immediately preceding the onset of tumor regression. In other words, the suppressor T cells defined by the assay developed in this laboratory function to inhibit the induction and generation of a secondary antitumor immune response in tumor-bearing test recipients. This is in keeping with the additional finding (19) that, in order for Ly-1$^+$2$^-$ suppressor T cells to suppress adoptive immunity, they need to be given to the TXB recipients soon after giving donor memory T cells, well before a secondary adoptive response develops in the recipient, and before tumor regression commences. Suppressor T cells fail to interfere with tumor regression once it is in progress.

The fact that tumor-induced suppressor T cells bear the Ly-1$^+$2$^-$, L3T4a$^+$ membrane phenotype of helper T cells should no longer be considered peculiar. Suppressor T cells with the surface phenotype of helper cells have been shown to play a role in suppressing immunity in other models of antitumor immunity (28) and in mediating immunologic tolerance

to allografts (29). They have also been shown to be responsible for suppressing anti-*Leishmania* immunity in susceptible strains of mice (30). Whether they are the ultimate mediators of suppression, however, has yet to be determined.

Summary

It has been shown with several immunogenic tumors under study in this laboratory that $Ly2^+$ effector T cells, capable of passively transferring antitumor immunity, are progressively generated between days 6 and 9 of tumor growth and are then progressively lost. It has been shown, in addition, that the loss of effector T cells occurs as the host progressively acquires $Ly\text{-}1^+2^-$, $L3T4^+$ suppressor cells capable, on passive transfer, of suppressing the expression of adoptive antitumor immunity in TXB recipients. Because the curve for the production of suppressor T cells is the reciprocal of the curve for the loss of effector T cells, it is logical to hypothesize that acquisition of the former T cells causes the loss of the latter. Presumably, because of the nature of tumor antigens, suppressor T cell production is evoked after an immunogenic tumor grows beyond a certain critical size. Therefore, in order to cause the regression of an established tumor by passive transfer of reasonable numbers of tumor-sensitized T cells from immunized donors, it is necessary to immunodepress the recipient to prevent it from producing suppressor T cells. With this knowledge, it is now possible to demonstrate successful adoptive immunotherapy of established tumors in a routine fashion.

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Role of Concomitant Antitumor Immunity in Restraining Tumor Metastasis

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Primary Tumor Growth Restrains Tumor Metastasis

Clinical and experimental studies (1–4) have shown that surgical treatment of cancer is often not curative because it fails to prevent the growth of metastatic cancer cells that have disseminated to other parts of the body. In some cases, an apparent disease-free interval may elapse before tumor metastases are detected. In other cases, however, excision of the primary tumor may actually enhance the growth of already seeded secondary tumor deposits (1, 3, 5–7), thus indicating that the primary tumor in some way retards the growth of metastases. For example, in studies on the postoperative development of metastases in mice bearing spontaneously metastasizing tumors (1, 2, 5–7), it was found that although excision of the primary tumor inhibited the development of additional metastases, it caused those metastases already seeded to grow more rapidly. Similar findings have been reported in hamsters bearing a lymphoblastic lymphoma (8), suggesting that the inhibitory effect of a primary tumor on the development of distant metastases is not limited to murine tumor systems.

Nonimmunologic Mechanisms

The mechanisms responsible for the accelerated growth of metastases after primary tumor excision remain unclear. Nonimmunologic, as well as immunologic factors have been proposed. DeWys (9) noted that as a tumor grows larger, its rate of growth, and that of a secondary tumor, decrease simultaneously. After removal of the primary tumor, however, the secondary tumor increased its rate of growth. Moreover, because the tumor-bearing host was unable to inhibit an implant of its own tumor cells, this author proposed that nonimmunologic mechanisms associated with the "total body tumor burden" were responsible for the reduced rate of tumor growth in animals bearing large tumors. He reasoned that reducing the "total body tumor burden" either made available more

nutrients for the remaining tumor deposits, or resulted in reduced levels of growth-inhibitory factors. In a later study, Gorelik et al. (10) also postulated the existence of a tumor-induced, nonimmunologic inhibitory factor that inhibits growth of a second tumor implant, and implied that this factor decayed in mice after removal of the primary tumor mass. Neither study, however, was able to recover the putative inhibitory factor from the serum and show that it could inhibit tumor growth in vitro, or in vivo. Therefore, it is reasonable to state that the role of tumor-induced, nonimmunologic factors in restraining tumor metastasis remains speculative.

Immunologic Mechanisms

On the other hand, the proposal that immunologic mechanisms might account for the inhibitory effect of a primary tumor on the growth of secondary tumor deposits is based on more convincing evidence. It is known, for example, that progressive growth of an immunogenic tumor in its immunocompetent host results in the generation of a paradoxical state of concomitant immunity that can prevent the growth of a second tumor implant given at a distant site (11). There is evidence that concomitant immunity can be generated against autochthonous (12), as well as syngeneic transplantable tumors. That it is generated in humans is indicated by the results of a limited study (13) showing that cancer patients can reject grafts of their own tumors. That concomitant immunity may represent a fairly common response to progressive tumor growth is also suggested by the numerous reports (14–16) of the presence in the blood of tumor-bearing humans and animals of lymphocytes that are specifically cytotoxic for tumor cells in vitro. These in vitro findings, together with the evidence that concomitant immunity can be transferred to normal recipients with Thy-1.2-positive lymphocytes but not with serum (17), have been interpreted as representing evidence that concomitant immunity is cell-mediated. More recently, it has been shown with the Meth A fibrosarcoma (18) and the P815 mastocytoma (19) that progressive tumor growth results in the generation of Ly-1^+2^+ T cells, which are capable, on passive transfer, of causing tumor regression in vivo and of causing specific lysis of tumor cells in vitro. However, these studies (18, 19) also showed that concomitant immunity generated to these tumors decays after the primary tumor grows beyond a certain critical size. Thus, in the case of the P815 mastocytoma, and the Meth A fibrosarcoma, progressive tumor growth resulted in the generation of peak numbers of Ly-1^+2^+ effector T cells around day 9 of tumor growth. After day 9, however, the number of effector T cells decreased rapidly in association with the progressive

acquisition of Ly-1^{+}2^{-} suppressor T cells that are capable of inhibiting the expression of passively transferred immunity against an established tumor in T cell deficient (TXB) recipients (18, 19). On the basis of these findings, it was postulated that immunogenic tumors grow progressively in their immunocomponent hosts because the immune response they evoke is down-regulated by suppressor T cells before an adequate number of effector T cells is generated to destroy the tumor.

Another important host defense component that is generated as a consequence of progressive tumor growth is an activated macrophage system. Studies conducted with the SA-1 sarcoma revealed that tumor-bearing mice acquire a greatly enhanced macrophage-mediated capacity for resisting infection with the bacterial parasite *Listeria monocytogenes* (17). Peak levels of macrophage activation were found to be concordant with peak levels of concomitant immunity, suggesting that macrophage activation relies on a T cell-mediated mechanism of immunity. The importance of macrophages in inhibiting neoplastic growth is a well-documented phenomenon that has been demonstrated directly in vitro (20, 21) and indirectly in vivo (22, 23).

Even though the possession of activated macrophages and tumor-sensitized effector T cells appears to have no restrictive influence on the growth of a primary tumor (17–19), there is evidence that these components of concomitant immunity play an important role in retarding both the rate of spread and the growth of tumor metastases. For example, if concomitant immunity functions as an important mechanism for inhibiting the spread of metastatic tumor cells and the growth of tumor metastases, it follows that any procedure that interferes with the generation of this immunity, or causes it to decay at a faster rate, should result in a more rapid development of systemic disease. Indeed, there is published evidence which shows that immunosuppression caused by exposure to ionizing radiation (24), treatment with cytotoxic drugs (25), or T cell depletion (26–28), renders tumor-bearing mice more susceptible to spontaneous metastases, and non-tumor-bearing mice much more susceptible to the growth of metastases artificially induced by intravenous infusion of tumor cells. In agreement with these findings, recent studies in this laboratory (29) showed that mice made T cell-deficient by thymectomy and whole-body gamma radiation and reconstituted with syngeneic bone marrow (TXB mice) were incapable of generating concomitant immunity in response to growth of the P815 tumor. The consequences of this were not seen in the growth rate of the primary P815 tumor in both normal and TXB mice, but instead, were seen in their susceptibility to systemic disease. Thus, although an intradermal implant of 2

$\times 10^6$ P815 cells grew at a similar rate in normal and TXB mice, TXB tumor-bearing mice died 2 weeks earlier. Evidence to support the idea that TXB tumor-bearing mice died sooner because they were more susceptible to systemic disease was obtained by determining the proportion of TXB mice versus normal mice that eventually died of metastatic disease after excision of their primary intradermal tumors at different times of tumor growth. These studies (29) showed that as early as day 4 of tumor growth, 80% of TXB tumor-bearing mice were destined to die from metastases that had already seeded the draining lymph node and spleen, and that by day 6 of tumor growth 100% had lethal metastases. In contrast, only 10% of normal mice died from metastases after excision of a day 6 tumor. Moreover, it was not until after day 9, when concomitant immunity had decayed, that 50–80% of the immunocompetent mice eventually died from metastases after excision of their day 12 and day 16 tumors. These findings support the idea, therefore, that the capacity of normal mice to generate a short-lived state of concomitant immunity in response to progressive growth of the P815 tumor temporarily halts the development of systemic disease and prolongs host survival.

Enumeration of Metastatic P815 Cells with an In Vivo Biological Assay

Although the foregoing studies clearly showed that TXB tumor-bearing mice are more susceptible to systemic disease, they were based on survival times and not direct evidence that failure to generate concomitant immunity resulted in the accelerated appearance and growth of metastatic P815 cells in the draining lymph node and spleen. Thus the evidence is correlative and does not provide a direct cause-and-effect relationship. In order to determine how concomitant immunity functions to inhibit tumor metastasis, it was considered necessary to enumerate the actual number of metastatic tumor cells in the organs of a tumor-bearing host at different times of tumor growth. With this type of information, it could be determined whether the generation of a state of concomitant immunity serves to inhibit tumor metastasis by delaying the appearance of metastatic P815 cells in the draining lymph node and spleen, or instead, by destroying a significant proportion of already seeded metastatic P815 cells at these sites once peak levels of immunity are generated.

Numerous procedures have been described in the literature for quantifying tumor metastases in the blood and internal organs of tumor-bearing hosts (30–36). Many of these procedures, however, lack the sensitivity and precision that are required to enumerate small numbers of metastatic

tumor cells in the presence of large numbers of host cells. For this reason a mouse survival assay, based on the finding that the survival time of mice is inversely related to the number of tumor cells injected intraperitoneally, was investigated in this laboratory (37) for its suitability as a procedure to enumerate metastatic tumor cells that have disseminated from a primary intradermal P815 tumor to the draining lymph node and spleen. In preliminary experiments a standard reference curve, relating the mean survival time of mice to the number of P815 cells injected intraperitoneally, was prepared by injecting groups of mice intraperitoneally with graded numbers of P815 cells. It was found that the mean survival time was a linear function of the $\log_{10}$ number of P815 cells injected (37). In addition, it was found that as few as 10 P815 tumor cells could be detected with this assay. In order to enumerate metastatic P815 cells in the organs of a tumor bearing host, single-cell suspensions were prepared with lymph nodes or spleens from tumor-bearing mice, and equal aliquots injected into different groups of five test recipients. The mean survival times of the test mice were determined and used to estimate tumor cell number in the cell suspensions from the standard reference curve.

However, because mice bearing a progressive P815 tumor acquire T cells in their draining lymph nodes and spleens that are capable of lysing P815 cells in vitro (19, 37), it was considered necessary to remove host cells from cell suspensions of these organs before injection into test mice, so that the actual number of P815 cells in the cell suspensions would not be underestimated. This was accomplished by performing the studies in semisyngeneic F1 B6D2 mice and by employing an anti-H-2^b antiserum to destroy H-2^{bxd} host cells without affecting H-2^d P815 cells. It was found that treatment of cell suspensions with anti-H-2^b serum and complement destroyed greater than 90% of B6D2 lymph node cells and 85–90% of spleen cells without significantly affecting P815 tumor cells (unpublished). Preliminary studies (37) using this method to deplete host cells from lymph node and spleen cell suspensions revealed that significantly higher numbers of metastatic P815 cells could be counted in treated- than untreated-cell suspensions from a tumor-bearing host. In addition, it was shown that if lymph node or spleen cells from a tumor-bearing host were mixed with a standard number of P815 cells and injected intraperitoneally into mice, the host cells were able to destroy P815 cells in a dose-dependent fashion. However, if the cell suspensions were treated with anti-H-2^b serum and complement before injection into test mice, the antitumor function of the host cells was ablated. Additional experiments showed that the host cells capable of destroying P815 cells in test mice were Ly-1^+2^+ T cells, and that their acquisition coincided with the generation of systemic con-

comitant antitumor immunity. On the basis of these findings, cell suspensions, to be analyzed in the mouse survival assay were routinely treated with anti-H-2^b serum and complement before injection into test mice.

Kinetics of Appearance and Growth of Metastatic P815 Cells in the Organs of a Tumor-bearing Host

Using the modified (anti-H-2^b) mouse survival assay, experiments were designed to follow the kinetics of appearance and growth of metastatic P815 cells in the draining lymph nodes and spleens of normal and TXB tumor-bearing mice at different times of tumor growth (29). The purpose of these experiments was to determine whether metastatic P815 cells appeared earlier, or increased in number more rapidly, in the draining lymph node and spleen of a TXB tumor-bearing mouse, which was unable to generate concomitant immunity. The experiment involved injecting groups of normal and TXB mice intradermally on the upper right flank with 2×10^6 P815 cells. This site was chosen because the lymphatic drainage from the tumor site is primarily to the axillary lymph node. At different times of tumor growth, five mice from each group were killed and the number of P815 cells in the draining lymph nodes and spleens was determined with the mouse survival assay. It was found that during the first 6 days of tumor growth, the rate of increase in the number of metastatic P815 cells was similar in the draining lymph nodes and spleens of normal and TXB tumor-bearing mice. However, during the generation of concomitant immunity between days 6 and 9 of tumor growth the number of metastatic P815 cells declined approximately 90% in the organs of immunocompetent tumor-bearing mice. Thereafter, the number of tumor cells in their nodes and spleens remained essentially unchanged until day 16 when the number of tumor cells at these sites increased concordantly with the decay of concomitant immunity. In contrast, there was no decrease after day 6 in the number of metastatic P815 cells in the draining lymph nodes and spleens of TXB tumor-bearing mice. Instead, there was a progressive increase in the number of tumor cells at these sites, which caused these mice to die much sooner than immunocompetent tumor-bearing mice. Overall, the results from this study showed that in an immunocompetent host there is a triphasic pattern of metastasis of P815 cells from a primary intradermal site to the draining lymph node and spleen. Thus, an initial period of progressive increase in the number of metastatic P815 cells during the first 6 days of tumor growth is followed by a period during which the number of metastatic cells decreases or remains unchanged between days 6 and 16

of tumor growth, and a final period during which the number of metastatic cells again increases progressively after day 16.

The triphasic pattern of metastasis of the P815 tumor in an immunocompetent host was confirmed using a soft agar colony assay as an alternative procedure for enumerating P815 tumor cells in the draining lymph nodes and spleens of tumor-bearing mice. The principle advantage of this procedure over the mouse survival assay is that colonies in soft agar can be counted microscopically within 7–10 days, instead of having to wait up to 21 days for test mice to die in the mouse survival assay. The results from preliminary experiments with the soft agar assay (Table I) showed that the colony-forming efficiency of the P815 tumor in soft agar was approximately 30–50%. In addition, it was found that the presence of host cells from the lymph node and spleen of a tumor-bearing host had no significant effect on the colony-forming efficiency of the P815 tumor in soft agar. Therefore, unlike the mouse survival assay, it was not necessary to treat cell suspensions with anti-H-2^b serum and complement before plating in soft agar. However, because the soft agar assay was unable to detect small numbers of P815 cells in the presence of large numbers of host cells owing to cell crowding, cell suspensions, on some occasions, were treated with anti-H-2^b serum and complement in order to improve the sensitivity of the assay. This treatment selectively depleted H-2^{bxd} host cells (85–90%) so that as few as 10 P815 cells could be enumerated in the lymph node and spleen of a tumor-bearing host.

The results shown in Figure 1 compare the ability of the mouse survival assay and the soft agar colony assay to enumerate metastatic P815 cells in the organs of a tumor-bearing host. Lymph node and spleen cell

Table I. Effect of Day 9 Spleen Cells on Plating Efficiency of P815 Tumor

No. P815 cells/dish	*No. spleen cells/dish*	*Colonies*[a]	*PE*[a]
	× 10⁴		*%*
0	100	0	0
250	0	102 ± 5	41 ± 2
250	100	110 ± 7	44 ± 3
250	25	105 ± 8	42 ± 3
250	5	115 ± 5	46 ± 2

a. Data are mean ± SEM (n = 3).

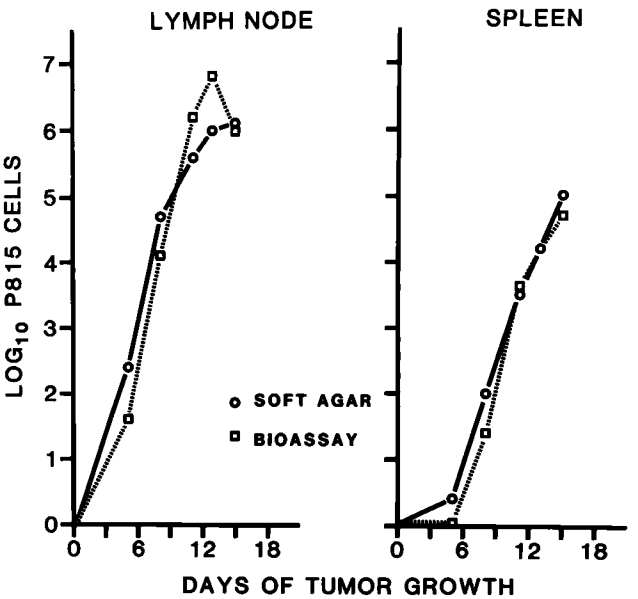

Figure 1. Evidence that the mouse survival assay and the soft agar colony assay give comparable results when used to enumerate metastatic P815 cells in the draining lymph node and spleen of a TXB host with an intradermal P815 tumor. Lymph node and spleen cell suspensions were prepared from five tumor-bearing mice at the times indicated and a portion of each either plated in soft agar or injected intraperitoneally into test mice after treatment with anti-H-2^b serum and complement. The results show that both procedures counted similar numbers of P815 cells.

suspensions, from TXB mice bearing an intradermal P815 tumor, were prepared at different times of tumor growth, and a portion of each either plated directly in soft agar or treated with anti-H-2^b serum and complement before injection into the peritoneal cavities of test mice. The number of P815 cells in each cell suspension was determined both by counting colonies in soft agar, and from the mean survival times of test mice. It can be seen that the two procedures agreed well in terms of their capacity to enumerate metastatic P815 cells in the organs of a tumor-bearing host.

Based on these findings, the soft agar colony assay was used as an alternative procedure for investigating the kinetics of appearance and growth of metastatic P815 cells in the draining lymph nodes and spleens of mice bearing an intradermal P815 tumor. Groups of normal and TXB mice were injected intradermally on the upper right flank with 2×10^6 P815 cells. At different times of tumor growth cell suspensions were prepared from the draining lymph nodes and spleens of three mice from each group.

The cell suspensions were serially diluted and plated in soft agar. The results from this experiment are shown in Figure 2 where it can be seen that in both normal and TXB tumor-bearing mice there was an initial period of progressive accumulation of P815 cells in the draining lymph node and spleen until days 6–8 of tumor growth. By day 6 there were 10^3 P815 cells in the lymph nodes of both types of mice, and by day 8 there were 10^2 P815 cells in their spleens. In immunocompetent tumor-bearing mice this was followed by a period between days 6 and 16 of tumor growth when the number of P815 cells at these sites remained essentially unchanged. This period coincided with the generation of a short-lived state of concomitant immunity in immunocompetent, tumor-bearing mice (19, 37). After day 16, however, when concomitant immunity had decayed, the number of P815 cells in the draining lymph nodes and spleen again increased progressively. On the other hand, it can be seen that TXB tumor-

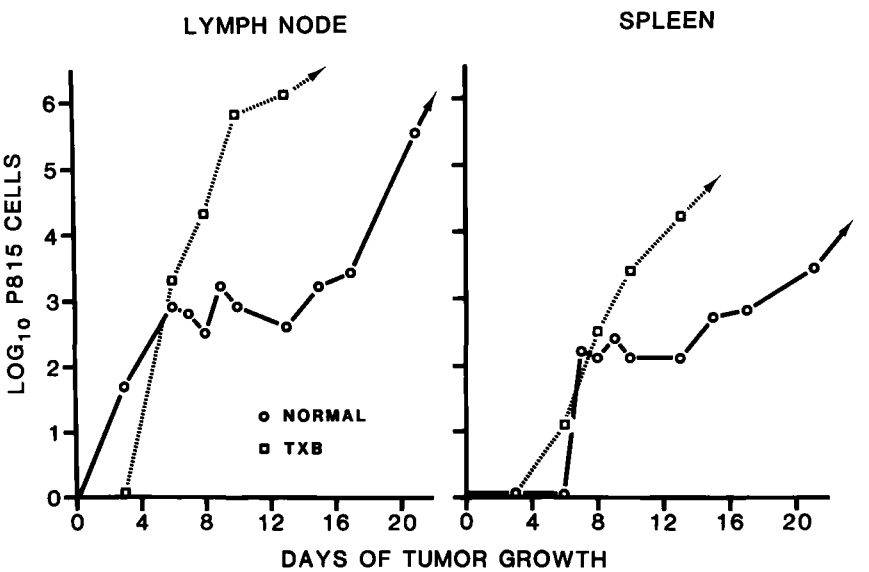

Figure 2. Kinetics of appearance and growth of metastatic P815 cells in the draining lymph node and spleen of normal and TXB mice bearing an intradermal P815 tumor. The number of metastatic tumor cells in the draining lymph node (*left*) and spleen (*right*) of tumor-bearing mice was determined at the times indicated with a soft agar colony assay. The results show that the number of metastatic P815 cells in the draining lymph node and spleen of an immunocompetent tumor bearer remained essentially unchanged between days 6 and 16 of tumor growth. In contrast, the number of metastatic tumor cells in a TXB tumor bearer increased progressively without evidence of any change in the rate of increase. Each point represents the mean number of tumor cells enumerated in the lymph nodes or spleens of three tumor-bearing mice.

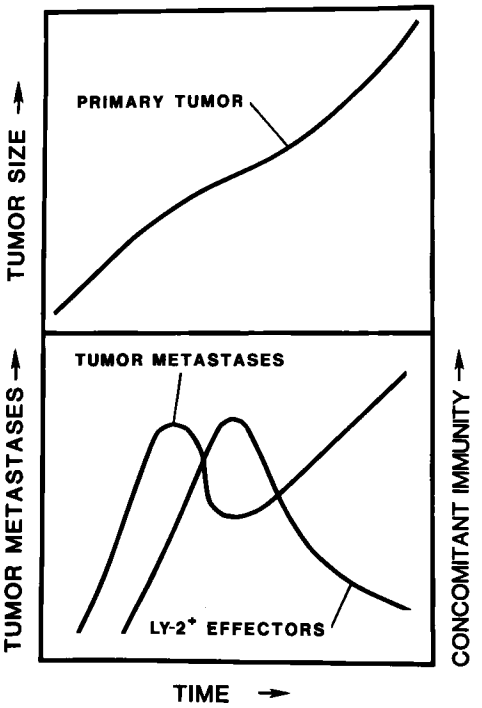

Figure 3. Diagrammatic representation of the relationship between the kinetics of appearance and growth of metastatic P815 tumor cells in the organs of a tumor-bearing host and the generation of a short-lived state of T cell-mediated concomitant antitumor immunity. The diagram is meant to show that during progressive growth of an intradermal P815 tumor the number of metastatic P815 cells in the organs of a tumor-bearing host is inversely related to the number of Ly-2⁺ effector T cells.

bearing mice were unable to retard the progressive accumulation of P815 cells in their draining lymph nodes and spleens. Thus, these findings confirm those obtained originally with the mouse survival assay (29) showing that in mice competent to generate concomitant immunity, there is a triphasic pattern of appearance and growth of P815 cells in the draining lymph node and spleen.

The foregoing findings can be represented diagrammatically as shown in Figure 3 where a curve representing the triphasic pattern of tumor metastasis is superimposed on the curve for the generation and decay of concomitant immunity as measured by the production of Ly-2⁺ cytolytic T cells (19, 37). The diagram is meant to show that during progressive growth of an intradermal P815 tumor there is a reciprocal relationship between the number of metastatic P815 cells in the organs of a tumor-bearing host and the number of Ly-2⁺ effector T cells. This finding provides strong evidence to support the idea, therefore, that the generation of a state of concomitant immunity functions as an important mechanism of defense against tumor metastasis.

Evidence That T Cell-mediated Concomitant Immunity Restrains Tumor Metastasis

To provide direct evidence that concomitant immunity is the mechanism responsible for temporarily halting and reversing systemic disease in an immunocompetent tumor-bearing host, an experiment was designed to determine whether passively transferred spleen cells from concomitant immune donors would prolong the survival time of TXB tumor-bearing recipients by destroying metastatic P815 cells. The experiment involved infusing 1.5 × 10^8 spleen cells, from donors bearing a 9-day P815 tumor, intravenously into TXB recipients bearing a 4-day intradermal P815 tumor. Controls consisted of TXB tumor-bearing mice infused with the same number of normal spleen cells and TXB mice that received no spleen cells. The results in Figure 4 (*left panel*) show that passively transferred spleen cells from donors at the peak of concomitant immunity had only

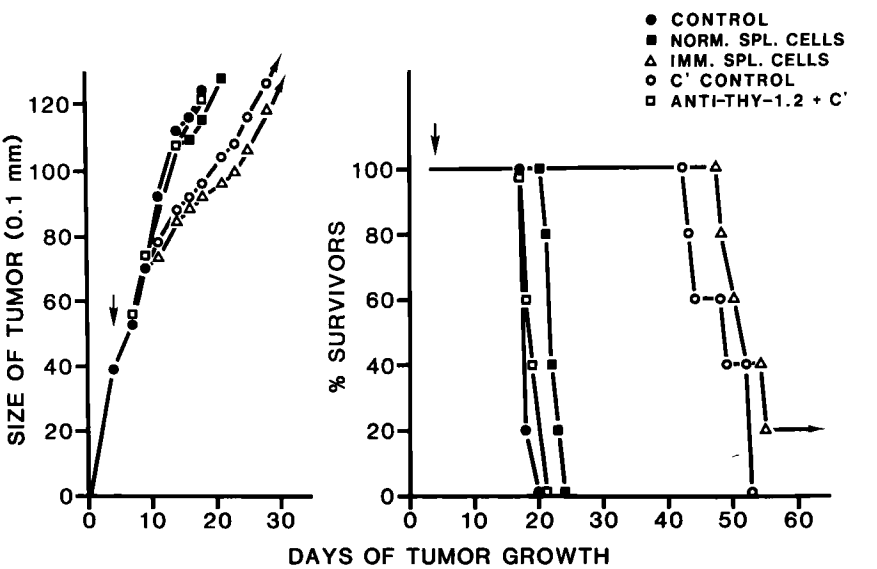

Figure 4. Evidence that spleen cells from donors at the peak of concomitant immunity are capable, on passive transfer, of significantly prolonging the survival time of TXB tumor-bearing recipients (*right*), even though they have only a small effect on the growth of the primary tumor (*left*). Spleen cells (1.5 × 10^8) from donors bearing a day 9 tumor were injected intravenously into TXB recipients with a day 4 tumor. Immune spleen cells treated with Thy-1.2 antibody and complement (C), and normal spleen cells, both failed to prolong the mean survival time of TXB recipients. Tumor measurements and percent survivors are based on five mice per group.

a small effect on progressive growth of the primary tumor in TXB recipients. In spite of these findings, however, the results in Figure 4 (*right panel*) show that spleen cells from concomitant immune donors were capable, on passive transfer, of prolonging the mean survival time of the TXB tumor-bearing recipients by nearly 4 weeks. In contrast, an infusion of spleen cells from normal donors had no significant effect on the survival time of TXB tumor-bearing recipients. These findings, together with the additional finding that treatment of the donor spleen cells with Thy-1.2 antibody and complement completely abolished their capacity to prolong the survival of TXB tumor-bearing recipients, provide good evidence that concomitant immunity functions to prolong the survival of TXB tumor-bearing mice not by inhibiting growth of the primary tumor, but, instead by temporarily halting and/or reversing systemic disease.

Additional evidence to support the idea that concomitant immunity retards tumor metastasis was provided by studies (29) in which the mouse survival assay was used to enumerate the number of metastatic P815 cells in the organs of TXB tumor-bearing mice adoptively immunized on day 4 of tumor growth with 1.5×10^8 spleen cells from concomitant immune donors. These studies showed that adoptive immunization with spleen cells from concomitant immune donors caused a rapid decline in the number of metastatic P815 cells in the draining lymph nodes of TXB tumor-bearing mice, and significantly delayed the appearance of metastatic cells in their spleens. These studies provided a direct demonstration, therefore, that spleen cells from a donor at the peak of concomitant immunity are capable, on passive transfer, of mediating the destruction of metastatic P815 cells in a tumor-bearing host.

Summary

In conclusion, these findings show that progressive growth of the P815 mastocytoma in an immunocompetent host results in the generation of a T cell-mediated state of concomitant immunity. The immunity is mediated by Ly-1^+2^+ effector T cells that are present around day 6 of tumor growth, reach peak levels on day 9, and decay progressively thereafter. The decay of immunity is associated with the progressive acquisition of Ly-1^+2^- supressor T cells that down-regulate the production of effector T cells before enough are generated to cause tumor regression. This may explain why immunogenic tumors are able to grow progressively in an immunocompetent host.

Although concomitant immunity fails to prevent progressive growth of a primary tumor, the experimental evidence discussed in this chapter shows that this host defense mechanism temporarily restrains tumor metastasis and prolongs host survival. By measuring the actual numbers of metastatic tumor cells in the organs of a tumor-bearing host with a mouse survival assay and a soft agar colony assay, it was shown that metastatic tumor burden is inversely related to the strength of concomitant immunity. Thus, before the generation of peak levels of immunity on day 6 of tumor growth the metastic tumor burden increased; it decreased during the generation of peak levels of immunity between days 6 and 9; and it increased again after day 12 as immunity decayed. It was shown, in addition, that if steps are taken to prevent the generation of concomitant immunity, the host dies much sooner, because of the more rapid dissemination and growth of tumor cells throughout its body. This evidence together with the finding that spleen cells from concomitant immune donors were capable, on passive transfer, of destroying already seeded metastic tumor cells and preventing further tumor cell dissemination suggest that concomitant immunity is an important host defense mechanism, the preservation of which needs to be considered when planning to treat tumors by survery and/or chemotherapy.

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Use of Antibodies Directed against the T Cell Receptor to Focus the Effector Activities of T Cells

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Introduction

The receptor on the surface of T cells that binds foreign antigen in association with self major histocompatibility complex (MHC) antigens has recently been identified by using monoclonal antibodies (1, 2). The receptor is a two-chain disulfide-bonded heterodimer, both the α- and β- chains of which possess variable and constant region sequences. This heterodimer is physically linked on the T cell surface with a complex of three polypeptides referred to as the T3 complex (3).

Monoclonal antibodies directed at sites on this T cell receptor-T3 complex can all inhibit the function of the T cells that they recognize. This is the case whether the antibodies are directed against idiotypic determinants expressed on the heterodimer, or against an allotypic determinant on the β-chain, or against the T3 complex. In the latter case, all T cells are bound and inhibited. These same antibodies can be used to mimic the effects of antigen recognition. For example, they can induce interleukin 2 secretion or immune interferon secretion, and under appropriate conditions, can serve as mitogens to stimulate T cells to proliferate.

Recently, it was shown that antireceptor antibodies could be used to target sites for the delivery of effector T cell functions. It was shown initially that a B cell hybridoma producing an anti-idiotypic antibody directed against a cytotoxic T lymphocyte (CTL) clone was a sensitive target for lysis by the recognized CTL (4). This was despite the fact that the B cell hybridoma did not express the MHC antigens that were nominally recognized by the CTL clone. The suggestion was that the antibody-combining site on the surface of the B cell is responsible for bringing the CTL to that site, thus activating the lytic mechanism leading to the destruction of the cell. Similarly, it was subsequently shown that all of the murine B cell hybridomas producing antibodies against the T3 complex are sensitive targets for lysis by any human CTL clone (5). The

hypothesis that the antireceptor-combining site serves as a target structure for CTL lysis was confirmed by covalently linking antireceptor antibodies to various cell types and showing that this converted them to sensitive CTL targets (6, 7).

We realized that antireceptor antibodies could potentially be used to specifically target certain cell types for attention by T cells. In order to do this, one would have to link the antireceptor antibody to another antibody specific for the cell type that one wished to target. This has turned out to be easily demonstrable in in vitro experiments because we and others (7, 8) have shown that chemically synthesized heteroconjugates of monoclonal antibodies in which one site is anti-T cell receptor, or anti-T3, and the other site is directed against any other chosen surface antigen (such as Thy-1.1, viral proteins, or trinitrophenyl), can focus T cell activity on a cell type identified by the second targeting antibody. In this fashion, one can combine the great advantages of monoclonal antibodies—that is to say, their availability and specificity—with the greater powers of T cell immunity in rejecting unwanted cells. Monoclonal antibody technique has rekindled interest in the possible use of "magic bullets" to eliminate unwanted cells. However, there is some reason to believe that T cell immunity is more effective in rejecting allogeneic grafts, tumor grafts, or virally infected cells. Although T cell immunologists have their own equivalent of monoclonal antibodies in antigen-specific T cell clones, these suffer from some disadvantages that are not applicable to antibodies. That is to say, the specificity of T cells is not just for foreign antigen but for foreign antigen seen in association with a particular MHC antigen, i.e., their specificity is MHC restricted. The second difficulty in using transplanted T cell activity is the fact that T cells themselves bear the strong histocompatibility antigens so that in most circumstances they could not be transplanted across a barrier.

It is our hope that in cases of cancer or viral infections that have escaped the normal rejection process by the host—for example, if the T cell system is blind to them—the tumor cells or the virally infected cells could be targeted for T cell attack by a hybrid antibody which recognizes a common determinant on a fraction of or on all T cell receptors plus a virus-specific or tumor-associated antigen.

The results that follow are in vitro demonstrations of how effectively antireceptor antibodies serve as targeting devices when they are attached to target cells either chemically, or as chemically constructed antibody heteroconjugates, or in the form of a true hybrid antibody secreted by a hybrid hybridoma cell line.

Results

Antireceptor Antibodies Serve as Target Structures for Cytolytic T Lymphocytes

Our work began with a report by Lancki and Fitch (4), which showed that a B cell hybridoma producing a monoclonal antibody (MAb) specific for an idiotypic determinant on a CTL clone served surprisingly as a target for cytolysis by that clone. The B cell hybridoma did not bear the histocompatibility antigens that the CTL clone recognized, and they hypothesized, quite reasonably, that the combining site on the MAb at the surface of the B cell hybridoma was responsible for focusing the CTL on that cell leading to its lysis. We decided to investigate whether this interpretation was correct by chemically coupling the surface of various cells with anti-T cell receptor MAbs and checking whether this rendered them as sensitive targets for CTL that expressed receptors bearing determinants recognized by such fixed antibodies.

Table I describes the properties of the CTL clones and MAbs that we have used in this work. All three CTL clones derive from H-2^b mice and specificity for H-2^d-encoded antigens. Most importantly for this work, none of the clones recognizes or lyses H-2^k-bearing target cells. The MAbs used are also listed in Table I. The anti-idiotypic MAb, F9, recog-

Table I. Cytolytic T Lymphocyte Clones and Monoclonal Antibodies

CTL clones
CTL Clone G4: from a BALB.B (H-2^b mouse, specific for H-$2D^d$ targets [9]).
CTL Clone OE4: from a B6 (H-2^b) mouse, specific for H-2^d targets (O. Kanagawa, unpublished).
CTL Clone OE25: from a B6 (H-2^b) mouse specific for H-2^d targets (O. Kanagawa, unpublished).
Antibodies
MAb F9: a clonotypic (idiotype-specific) IgG1, prepared in BALB/c against CTL clone G4 (9).
MAb F23.1: An IgG2a prepared from a C57L/J immunized with BALB.B T cells, specific for an allotypic determinant on ~25% of T cell receptors, including CTL clone OE4 (10).
MAb 20.8.4: an IgG2a prepared in C3H, specific for H-2^b (11).
MAb 19E12: an IgG2a, specific for the Thy-1.1 alloantigen (12).

Table II. Specific Lysis of Monoclonal Antibody-coupled Targets

Effector CTL clone	*S.AKR*	*S.AKR-20.8.4*	*S.AKR-F9*	*S.AKR-F23.1*
	% specific lysis, 4 hour ^{51}Cr-release assay			
G4 (10:1)[a]	6	2	38	3
OE4 (3:1)[b]	5	4	3	68

a. The T cell receptor of G4 (H-2^b anti-H-2^d) is F9 idiotype-positive, F23-allotype-negative (9).

b. The T cell receptor of OE4 (H-2^b anti-H-2^d) is F9 idiotype-negative, F23-allotype-positive (7).

nizes the heterodimeric T cell receptor of CTL clone G4 but does not recognize CTL clone OE4 nor OE25. The antireceptor MAb, F23.1, is specific for an allotypic determinant on the T cell receptor of CTL clone OE4 and does not recognize G4 or OE25.

S.AKR target cells, derived from AKR/J mice (H-2^k, Thy-1.1.), were coupled at their surface with various MAbs by using the heterobifunctional cross-linking reagent N-succinimydyl-3-(1-pyridyldithio) propionate SPDP (7). Table II shows that uncoupled S.AKR targets are not lysed by either CTL clone. However, when coupled with the appropriate antireceptor antibody, they become sensitive targets of the CTL clone recognized by the MAb. Thus, F9-coupled S.AKR targets are lysed by G4 CTL, whereas F23.1-coupled S.AKR cells are lysed by OE4 effector cells. Interestingly, when these S.AKR cells were coupled with an anti-H-2^b MAb, 20.8.4, they were not lysed by either clone. This is despite the fact that this MAb recognizes the H-2^b antigens expressed by the CTL clones and presumably brings the two cells together. Thus, it appears that there is a requirement that the CTL clone be contacted via its antigen receptor. This may reflect a need to activate the CTL or that the killing mechanism is tightly linked to the receptor. Similar results on the chemical coupling of antireceptor MAb to cells have recently been reported by Krantz et al. (6). Figure 1 shows a CTL/target titration of a similar experiment to the one reported in Table II.

To reinforce the argument that there are no requirements at the target cell level except that it must bear an antireceptor binding site, we studied

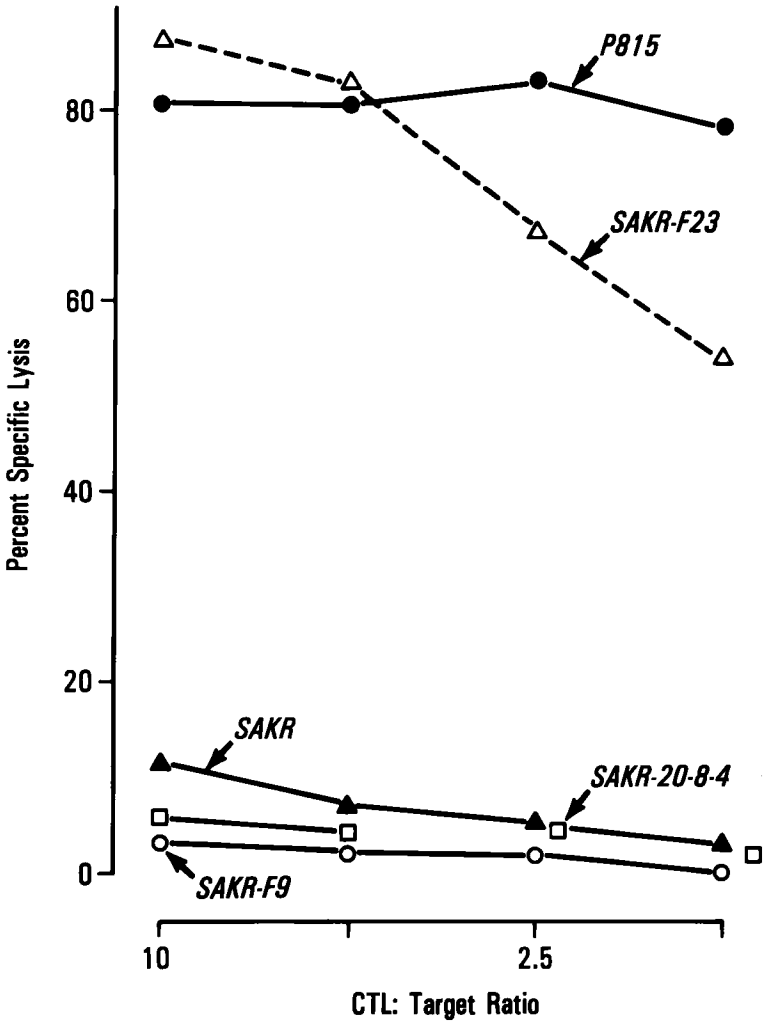

Figure 1. Anti-T cell receptor antibody coupled to the target cell surface renders the cell susceptible to T cell lysis. S.AKR target cells (H-2^k) were coupled with nothing (▲), F23.1 (△), F9 (○), or 20.8.4 (□), and tested for lysis by the F23.1-positive CTL clone OE4 (H-2^b anti-H-2^d). Unmodified target cells P815 (●) were specifically lysed.

the MAb-mediated lysis of lymphoma cells that express no class I or class II antigens. For this, we used the R1.TL$^-$ cell line derived by R. Hyman, which has lost the genes for β_2-microglobulin and thus does not express H-2 antigens (13). Table III shows quite clearly that the MHC-negative cell line, when chemically coupled with the F9 MAb, is a sensitive target for G4-mediated killing, and that when it is coupled with the anti-allotypic

MAb F23.1, it becomes a sensitive target for OE4-mediated killing. This experiment is really a more sophisticated version of the experiment that I reported with R. Hyman in 1977 in which we showed that H-2$^-$ cell lines could be lysed by CTL in the presence of an agglutinant such as PHA (13).

Heteroconjugates of Monoclonal Antibodies Can Specifically Target Sites for Attack by Cytolytic T Lymphocytes

After having shown that an antireceptor antibody can apparently fulfill all of the needs usually provided at the target surface by antigen-plus-MHC, we then wanted to know whether we could *specifically* target sites for attack by CTL. The most obvious antigen-specific targeting device is another MAb. Heteroconjugates of MAbs were constructed using SPDP (7) in which one site is anti-T cell receptor and the other site is directed against the Thy-1.1 alloantigen. Thy-1.1 was chosen as a target antigen because none of the CTL clones that we use express this allodeterminant, whereas there are many AKR/J-derived target cells that do. S.AKR (H-2^k, Thy-1.1) and EL4 (H-2^b, Thy-1.2) cells were ^{51}Cr-labeled and incubated with hybrid antibodies constructed as follows: 19E12 (anti-Thy-

Table III. Antireceptor Antibodies Covalently Linked to the Cell Surface Cause the T Cell-mediated Lysis of an H-2-negative Tumor[a]

Effector CTL clone[b]	*P815*	*R1.TL*$^-$	*F23.1-R1.TL*$^-$	*F9-R1.TL*$^-$
	% specific lysis of target cells[c]			
OE4	83	1	78	2
G4	75	0	− 1	40
OE25	81	0	1	1

a. The C58/J-derived, β_2-microglobulin-negative, H-2-negative cell line R1.TL$^-$ was reduced with dithiothreitol and coupled with SPDP-modified antibodies F23.1 (anti-allotype) or F9 (anti-idiotype).

b. The three CTL clones are derived from H-2^b mice and are specific for H-2^d antigens. OE4 expresses the F23.1 determinant on its receptor; G4 is F9 idiotype-positive; OE25 is not recognized by either antibody.

c. Effector cells were cultured with ^{51}Cr-labeled targets at a 30:1 ratio for 4 hours at 37°C.

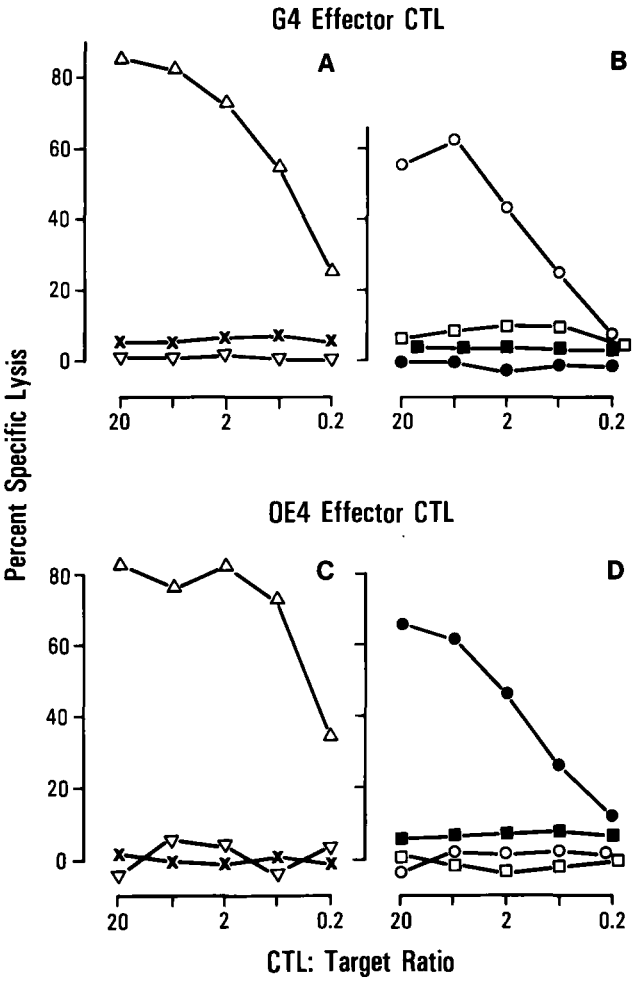

Figure 2. Heteroconjugates of monoclonal antibodies can specifically target cells for CTL-mediated lysis. Effector CTL clones were: (A and B) G4 (H-2^b anti-H-2^d), which is positive for the idiotypic determinant recognized by F9, and unreactive with F23; (C and D) OE4 (H-2^b anti H-2^d) CTL clone which is $F23^+$, $F9^-$. Target cells were: P815 (H-2^d, △); S.AKR (H-2^k, ▽); EL4 (H-2^b, X); S.AKR incubated with 19E12/F23 (●); S.AKR incubated with 19E12/F9 (○); EL4 incubated with 19E12/F23 (■); and EL4 incubated with 19E12/F9 (□).

1.1) linked to F9, and 19E12 linked to F23. After one wash, the cells were assayed for lysis by the CTL clones G4 and OE4 (Figure 2). The results showed that the Thy-1.1-positive lymphoma, S.AKR, was specifically targeted for lysis by the hybrid antibodies. Thus, the anti-Thy-1.1 site fixed the hybrid antibody to the surface of the lymphoma whereas the anti-T

cell receptor site focused the killer cell to act. EL4 (Thy-1.2) cells were *not* targeted for lysis. In other experiments, we have fractionated the cross-linked antibodies (19E12 × F23) by passage through a Sephacryl S-400 column. This revealed a major peak of targeting activity in the ~300,000 mol wt range with no activity in the 7S region or higher than 600,000 mol wt. By using such a purified preparation of heteroconjugated antibodies, we showed that the CTL could be coated or armed with the hybrid antibody and then function against the Thy-1.1-positive targets. Furthermore, the hybrid antibody can be incorporated directly into the mixture of CTL and targets with satisfactory results.

In order to show the general applicability of this technique, we have studied hybrid antibody-specific targeting in two other systems in vitro. In the first, we showed the EL4 cells infected with vesicular stomatitis virus could be targeted for lysis by CTL that were specific for H-2D^d antigens by an antireceptor-X-anti-G protein MAb heteroconjugate (Table IV). Finally, in collaboration with Ralph Reisfeld (Scripps Clinic and Research Foundation), we showed that the human melanoma cell line, M14, could be targeted for lysis by a murine CTL clone using a hybrid antibody constructed of antireceptor linked to MAb 9227, a melanocyte-associated specific antibody.

From these and other experiments with chemical coupling, we are confident that there are no special requirements for this type of antibody-

Table IV. Specific Targeting of Vesicular Stomatitis Virus-infected Cells by Monoclonal Antibody Heteroconjugates

		% Lysis at 13:1 E:T	
Target cells	*Hybrid antibody*	*G4CTL*[a]	*OE4 CTL*[b]
EL4	(F9 × I17)[c]	6	1
EL4	(F23 × I17)	1	2
EL4-VSV	(F9 × I17)	54	2
EL4-VSV	(F23 × I17)	6	31

a. CTL clone G4 (b anti-d) bears the idiotypic determinant recognized by F9 antibody.

b. CTL clone OE4 (b anti-d) bears the allotypic determinant recognized by F23.1 antibody.

c. The MAb I17 is specific for G protein of vesicular stomatitis virus (VSV) and was provided by L. Lefrancois.

directed T cell-mediated killing other than an antibody which reacts with the receptor complex becoming associated with the target cell surface.

A True Bivalent Hybrid Antibody Can Focus T Cell Activity

Because it is unlikely that chemical conjugates of two MAbs would be able to survive for very long in the circulation or penetrate very effectively to sites of inflammation, we decided to prepare bivalent, 7S hybrid antibodies. Preliminary experiments showed that recombinant $F(ab')_2$ frag-

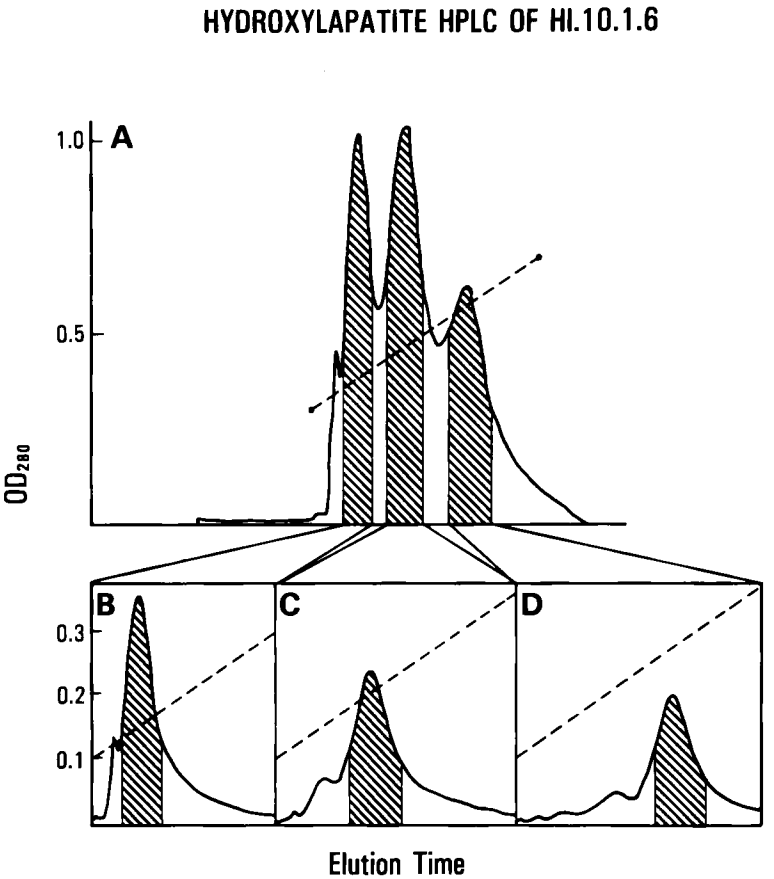

Figure 3. Fractionation of Protein A-Sepharose purified immunoglobulin produced by the hybrid hybridoma H1.10.1.6. The hydroxylapatite column was loaded in 10 mM sodium phosphate, pH 6.8, and eluted with a gradient of 50–350 mM sodium phosphate, pH 6.8. The three shaded fractions indicated in panel A were pooled, concentrated, and run under the same conditions: (B) peak I; (C) peak II; (D) peak III.

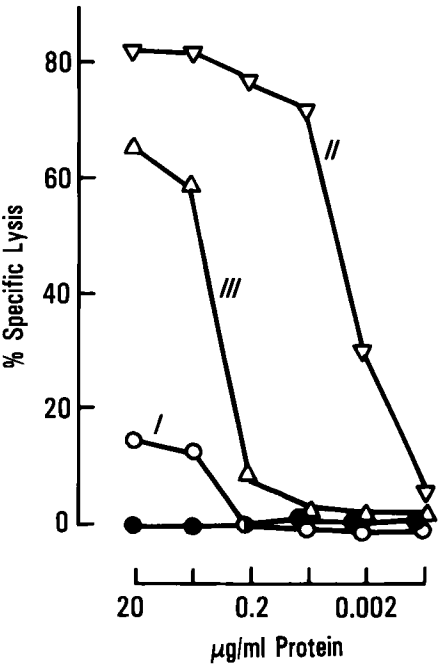

Figure 4. The three peaks from HPLC (Figure 3) were titered for their ability to cause the OE4-mediated lysis of ^{51}Cr-labeled S.AKR target cells.

ments prepared by reduction of F23.1 and 19E12 F(ab')$_2$ and reoxidation of a mixture of the two Fab' fragments were able to target Thy-1.1-expressing cells for lysis by T cells (unpublished observations). Next, we derived a hybrid hybridoma cell line, H1.10.1.6, which secretes large amounts of an F23.1/19E12 hybrid antibody.

A subline of F23.1 hybridoma was selected for resistance to 8-azaguanine (i.e., hypoxanthine-aminopterin-thymidine [HAT] sensitivity), and subsequently was grown in increasing concentrations of ouabain to 1×10^{-4} M. This HAT-sensitive, ouabain-resistant subline of F23.1 was fused to a HAT-resistant, ouabain-sensitive line of 19E12. Clones able to grow in HAT plus ouabain were selected and their supernatants were tested for the presence of both antireceptor (F23.1) activity and anti-Thy-1.1 (19E12) activity. We derived such a stable, double-producer hybrid hybridoma, H1.10.1.6, by repeated subcloning. Although the screening for both antibody activities was done in fluoresence assays, the culture supernatant of H1.10.1.6 was able to target S.AKR cells for lysis by CTL clone OE4 up to a dilution of 1:100. We produced ascites fluid from mice bearing

H1.10.1.6 and purified the IgG fraction by Protein A-Sepharose chromatography. Note that both parental antibodies are murine IgG2a. Fractionation of the IgG on hydroxylapatite high performance liquid chromatography (HPLC) is shown in Figure 3. Peak I, Figure 3B, coincides with the elution position of F23.1 and peak III, Figure 3D, with 19E12. Peak II is thus the putative hybrid antibody. Biochemical evidence in support of this was provided by isoelectric focusing (data not shown). Functional data consistent with the idea is shown in Figure 4. Peak I has little targeting activity for S.AKR targets and OE4 effector cells; peak III has some activity but only about 1% the activity of the most active peak II. Note that peak II can efficiently target S.AKR targets for lysis by a threefold excess of CTL at a protein concentration as low as 2 ng/ml.

Further experiments have shown that, in order for H1.10.1.6 IgG to function in this assay, (*a*) the target cell must express Thy-1.1, and (*b*) the CTL must express the receptor allotypic determinant recognized by F23.1.

Concluding Remarks

Hybrid antibodies can combine the great advantages of MAbs with T cell effector functions in destroying cells. The advantages of MAbs are their specificity, reproducibility, and availability in large amounts. T cells, on the other hand, are better able to reject unwanted cells from the body. We plan to use the hybrid antibody F23.1/19E12 secreted by the hybrid hybridoma cell line H1.10.1.6 to test the in vivo applications of this technique. An AKR/J lymphoma expressing Thy-1.1 will be inoculated in a Thy-1.2 host unable to reject it, and host T cells will be focused to the tumor site by the bispecific antibody.

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Immunotherapy of Cancer with Lymphokine-activated Killer Cells and Interleukin 2

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Introduction

The lymphokine-activated killer cell (LAK) represents a unique cell population capable of both in vitro and in vivo lysis of a broad spectrum of tumors. The in vitro LAK cell phenomenon was initially described when the incubation of murine splenocytes or human peripheral blood lymphocytes in media containing interleukin 2 (IL-2) resulted in the production of effector cells capable of lysing a wide variety of fresh, noncultured tumor cells in short-term ^{51}Cr-release assays. Normal adult fresh tissues were not lysed. The novel features of this anti-tumor activity were the broad range of tumor targets affected, the ability to lyse fresh, noncultured target cells from solid tumors (unlike natural killer cells), and the induction of killing by IL-2 alone without previous exposure to specific antigen (in contrast to classic cytotoxic lymphocytes). Interleukin 2 from several sources and species was able to activate LAK cells, and tumor lysis was not major histocompatibility complex (MHC) or even species-restricted.

Interleukin 2-induced nonspecific cytotoxicity, as seen in LAK cells, appeared to explain several previously described anomalous cellular cytotoxic activities. Lectin-stimulated cytotoxicity, antitumor cytotoxicity of cells generated in mixed lymphocyte reactions and by stimulation with other alloantigens, may all be mediated through the generation of IL-2.

After the in vitro studies of tumor lysis, efforts were directed toward therapy of animals bearing established, growing syngeneic tumors by adoptive immunotherapy using LAK cells. Because the generation and maintenance of LAK cells were absolutely dependent on the presence of IL-2, the treatment of tumor-bearing animals by the intravenous transfer of LAK cells was supported by systemic IL-2 administration. In a wide variety of murine sarcomas, melanomas, and carcinomas, metastatic to pulmonary or hepatic sites, therapy with LAK cells and IL-2 was shown to induce the regression of established metastases. Studies of the lungs

of animals after LAK cell and IL-2 therapy showed massive lymphocytic infiltration, and when lymphocytes were isolated from these lungs, they showed LAK activity in vitro. Animal treatment regimens were studied to optimize therapy and these therapeutic strategies are being used as the basis for the design of human protocols.

Phenomenon of the Lymphokine-activated Killer Cell

When lymphocytes were activated by lectins, in mixed lymphocyte culture or by a variety of other alloantigens, it was noticed that cellular cytotoxicity against tumor cells was generated (1–3). Subsequently, it was found that IL-2 was generated during these interactions, and that stimulation of lymphocytes with IL-2 containing media alone could generate cells with antitumor cytotoxicity (4–6); we termed this the lymphokine-activated killer (LAK) cell phenomenon. When murine lymphocytes are incubated with IL-2, sequential ^{51}Cr-release assays against the natural killer (NK)-resistant fresh tumor target MCA 102 sarcoma show the initial appearance of lysis after 24 hours and a rapid rise to maximal levels of lysis by 3–5 days (Table I). With human LAK cell generation, the findings are essentially the same. In all cases, tumor cell targets used in ^{51}Cr-release assays to assess LAK activity are prepared from either ascites or enzymatic digestion of fresh solid tumors and are uniformly NK-resistant.

Table I. Time Course of Recombinant Interleukin 2 Generation of Lymphokine-activated Killer Cell

Incubation in rIL-2 (hours)	*Lysis of MCA 102 Effector/target ratio*	
	50:1	*17:1*
	% ± SEM	
0	−3 ± 1	1 ± 1
24	18 ± 4	5 ± 2
48	42 ± 1	16 ± 5
72	44 ± 2	24 ± 2

Four-hour ^{51}Cr-release assay using the NK-resistant MCA 102 fibrosarcoma as the tumor target. Data represent the mean of quadruplicate values.

Table II. Lymphokine-activated Killer Cells Activated by Recombinant Interleukin 2 Kill a Variety of Fresh Natural Killer-resistant Tumor Targets

	LAK cell effector		*Fresh PBL effector*	
	Effector/target ratio			
Target cells	*40:1*	*10:1*	*60:1*	*15:1*
Fresh tumor				
Diagnosis				
Sarcoma (autologous)	76 ± 6	73 ± 1	−4 ± 10	−9 ± 1
Sarcoma	88 ± 3	78 ± 2	7 ± 2	−1 ± 1
Sarcoma	57 ± 3	48 ± 3	−8 ± 1	−12 ± 2
Sarcoma	85 ± 1	70 ± 5	9 ± 8	2 ± 5
Sarcoma	98 ± 1	87 ± 1	1 ± 2	1 ± 2
Sarcoma	64 ± 2	54 ± 7	−4 ± 3	−11 ± 8
Sarcoma	67 ± 3	57 ± 2	−6 ± 4	−3 ± 1
Colon Ca	62 ± 1	41 ± 3	−16 ± 1	−14 ± 1
Adrenal Ca	68 ± 2	41 ± 3	−16 ± 1	−20 ± 2
Esophageal Ca	78 ± 5	62 ± 2	0 ± 4	−1 ± 1
Pancreas Ca	28 ± 5	17 ± 2	−5 ± 1	−2 ± 1
Cultured tumor				
K562	105 ± 6	89 ± 2	46 ± 3	15 ± 1
Daudi	85 ± 5	101 ± 18	2 ± 2	3 ± 3

Adapted from Rayner et al. (7).

When cultured cell lines and NK-susceptible tumor cells are tested, they are uniformly lysed by LAK cells (Table II).

To demonstrate the broad specificity of LAK lysis, 41 consecutive human tumor preparations were tested for lysis by human LAK cells (7). Thirty-six preparations showed lysis of ≥10% at effector-to-target ratios of 20:1 to 80:1 (Table III). Some human fetal tissues and nontransformed cultured cells (e.g., concanavalin A [Con A] blasts) are occasionally lysed, but fresh adult cells are not lysed (8).

The initial studies of the generation of LAK cells used crude IL-2-containing supernatants from lectin-stimulated lymphocytes. When purified recombinant human IL-2 (rIL-2) became available after the expression of the JURKAT cell-line-derived IL-2 gene in *Escherichia coli* (9), it was possible to demonstrate that rIL-2 alone was sufficient to generate LAK activity. A variety of lymphokines and other biologic response modifiers, including interferon-gamma, were tested for either the ability to replace rIL-2 or improve on its efficacy in generating LAK. An absolute requirement for IL-2 was found and no combination was superior to optimal levels of rIL-2 (6, Chang, A. E., et al., manuscript in preparation).

The specific recognition site of LAK cells and the mechanism of target lysis are unknown. Preliminary work has shown that the generation of LAK activity correlates with the appearance of lytic granules also found in cytolytic T lymphocytes (CTL) and NK cells. (Henkart, P. A., private communication). The mechanisms of recognition and lysis represent a fertile area for future research.

Table III. Lymphokine-activated Killer Cells Kill a Variety of Fresh Human Tumor Targets

Diagnosis	*Number of specimens tested*	*Number killed by LAK cells (>10% specific lysis)*	*Lysis*	
			Mean	*(Range)*
Soft tissue sarcoma	20	18	49	(0–98)
Osteosarcoma	5	5	50	(24–65)
Adenocarcinoma				
Colon	7	4	20	(0–62)
Ovary	2	2	23	(15–31)
Pancreas	2	2	24	(20–28)
Lymphoma	3	3	41	(36–47)
Esophageal carcinoma	1	1	78	(78)
Adrenal carcinoma	1	1	68	(68)
Total	41	36		

Adapted from Rayner et al. (7).

Table IV. Generation of Lymphokine-activated Killer Activity from Thy-1.2$^-$ Murine Splenocytes[a]

Fraction/treatment	*Lysis of MCA 102*[b] *(LU_{30})*
Experiment 1	
C57BL/6 splenocytes	5.0
C57BL/10 SCN (nude) splenocytes	4.5
Experiment 2	
Untreated	30.9
C′-treated	11.4
Anti-Thy-1.2 + C′	12.9
Experiment 3	
Unsorted (FACS)	8.1
Thy 1.2$^-$	8.1
Thy 1.2$^+$	<2.0

Adapted from Yang et al., (10).

a. Splenocyte fractions are activated separately in rIL-2 for 3 days and assayed in 4-hour ^{51}Cr-release assay against fresh sarcoma target cells (MCA 102).

b. Lysis expressed as lytic units per 10^6 cells where one lytic unit is the number of cells required to lyse 30% of 10^4 target cells.

Characterization of the Lymphokine-activated Killer Cell In Vitro

The phenotype of the LAK cell precursor and activated effector cell has been investigated by several methods. In studies of the murine LAK precursor cell (Table IV), splenocytes depleted with anti-Thy-1.2 antibody and complement or splenocytes from nude mice generated normal LAK activity (10). In addition, when normal murine splenocytes were separated by fluorescence-activated cell sorting (FACS) into Thy-1.2$^-$ and Thy-1.2$^+$ cells and then activated in rIL-2, again Thy-1.2$^-$ cells generated normal LAK whereas Thy-1.2$^+$ cells generated inconsistent and usually lesser levels of cytotoxicity.

Depletion of Ia-bearing cells from whole splenocyte populations with anti-Ia antibody and complement (C′) did not interfere with LAK generation. When > 99% of Ia-bearing cells were removed by FACS separation, LAK generation was unimpaired, demonstrating no significant requirement for antigen-presenting cells in this process (Table V).

When antibody against the murine NK cell marker, asialo GM1, was used with complement to deplete normal splenocytes, the remaining cells showed a 90% decrease in the ability to respond to rIL-2 by producing LAK cells, but were fully capable of generating a classic CTL response (Figure 1). Therefore, the LAK precursor in murine spleen is Thy-1.2-negative, Ia-negative, and asialo GM1-positive.

After LAK effector cells have been generated by activation in rIL-2, they become susceptible to lysis by anti-Thy-1.2 antibody and C' (Table VI). The acquisition of Thy-1.2 antigen by LAK effectors is confirmed by FACS separation with nearly all lytic activity in the Thy-1.2$^+$ fraction. Ia-bearing cell depletion at the effector stage again failed to diminish LAK activity. Thus the LAK cell acquires the Thy-1.2 antigen, but remains Ia-negative after IL-2 activation.

Although no marker analogous to asialo GM1 exists for human lymphocytes, the human LAK precursor also appears to be a "null" cell lacking both B cell and T cell characteristics. The sequential depletion of human peripheral blood lymphocytes of nylon-adherent cells and then E-rosette-positive cells yields a small subpopulation of residual lymphocytes which generate 12-fold augmented LAK activity on a per-cell basis (Table VII). More stringent conditions adding T-cell depletion by anti-OKT3 antibody and C' and DR-bearing cell depletion with anti-DR antibody and

Table V. Generation of Lymphokine-activated Killer Activity from Ia- Murine Splenocytes[a]

Fraction/Treatment	*Lysis of MCA 102 (LU_{30})*
Experiment 1	
Untreated	30.0
C'-treated	11.4
Anti-Ia + C'	12.9
Experiment 2	
Unsorted (FACS)	1.0
Ia$^-$	1.0
Ia$^+$	$\leqslant$0.1

Adapted from Yang et al., (10).

a. All notation as in Table IV.

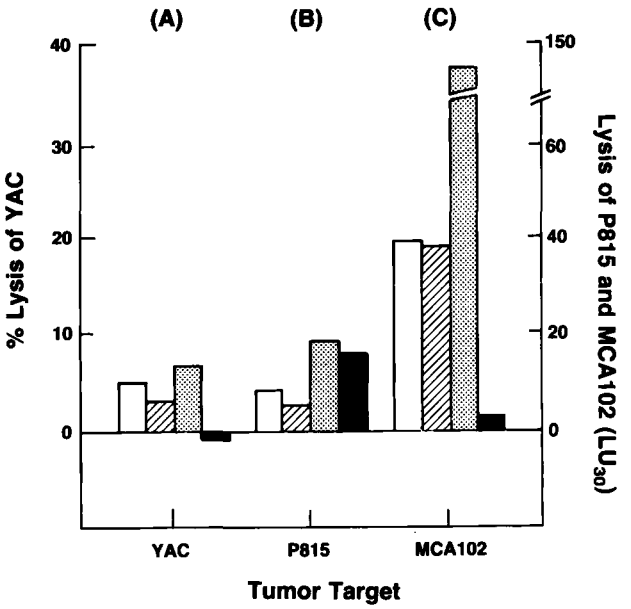

Figure 1. Normal splenocytes were treated with media ([□]), anti-asialo GM1 antibody only (▨), complement only (▩), or anti-asialo GM1 antibody and complement (■). They were then (A) assayed against YAC targets in ^{51}Cr-release assay (effector/target ratio = 100:1), (B) incubated with 2,000 rads-irradiated DBA splenocytes to generate CTL against H-2^{d} and assayed against P815 (H-2^{d}), or (C) activated for 3 days in rIL-2 1,000 U/ml and then assayed against the syngeneic, NK-resistant target MCA 102. YAC lysis is in percent lysis only; lysis of P815 and MCA 102 is expressed as lytic units per 10^{6} effector cells, where a lytic unit is defined as the number of effector cells necessary to lyse 30% of 10^{4} target cells in 4-hour ^{51}Cr-release assay.

C′ results in further enrichment for the LAK precursor cell. These results show the human LAK precursor phenotype to be analogous to the Thy-1.2^{-}, Ia^{-} phenotype of the murine LAK precursor cell. In murine and human systems, LAK cells are generated from the "null cell" population by IL-2 activation.

In Vivo Elimination of Established Metastases by Lymphokine-activated Killer Cells and Recombinant Interleukin 2

In order to assess the efficacy of LAK cells against tumor in vivo, we chose a murine model in which established, growing metastases from a variety of lethal syngeneic tumors were treated with systemic LAK cells and rIL-2 (11).

Tumor cells were prepared by the digestion of early-passage syngeneic methylcholanthrene-induced fibrosarcomas using collagenase, deox-

yribonuclease, and hyaluronidase. Then 3×10^5 cells were injected on day 0, and on day 3, when established pulmonary micrometastases were histologically demonstrable, the mouse was treated with an intravenous transfer of 10^8 LAK cells generated in vitro. Systemic rIL-2 was begun at that time and continued through day 8 and a second transfer of 10^8 cells was given on day 6. On day 14, the mice were killed and the lungs removed, insufflated with India ink, and bleached with Fekete's solution, and individual metastases were counted.

Treatment with low doses of systemic rIL-2 alone, LAK cells alone, or normal splenocytes with systemic rIL-2 did not significantly reduce the number of metastases when compared to animals treated with only Hank's balanced salt solution (HBSS) (Figure 2). The transfer of splenocytes cultured without rIL-2 (therefore without in vitro lytic activity) failed to reduce metastases even when accompanied by systemic rIL-2 adminis-

Table VI. Separation of the Lymphokine-activated Killer Effector Cell with Antibody and Complement or Fluorescence-activated Cell Sorting[a]

Treatment/fraction	*Lysis LU_{30}*
Experiment 1	
Untreated	16.2
C′ treated	13.1
Anti-Thy-1.2 + C′	1.3
Experiment 2	
Unsorted (FACS)	4.3
Thy 1.2^-	>10.0
Thy 1.2^+	⩽1.0
Experiment 3	
Unsorted (FACS)	12.4
Ia^-	12.0
Ia^+	<2.0

Adapted from Yang et al., (10).

a. Lymphokine-activated killer cells were generated from murine splenocytes by 3-day incubation in IL-2. They were then treated with antibody and C′ or subjected to FACS separation. Subpopulations of LAK effector cells were then assayed in 4-hour ^{51}Cr-release assay against the NK-resistant tumor target MCA 102. Other notation as in Figure 4.

Table VII. Separation of the Lymphokine-activated Precursor from Human Peripheral Blood Leukocytes[a]

PBL fraction	LU_{30}[b]
Unseparated	1.5
Nylon non-adherent	4.0
Nylon non-adherent, E-rosette-negative	18.5
Nylon non-adherent, E-rosette-positive	<1
Nylon non-adherent, E rosette-negative, $T3^-$, DR^-	>20[c]

Roberts, K., et al., unpublished observations.

a. Fresh peripheral blood leukocytes (PBL) obtained by leukapheresis were sequentially subjected to nylon adherence, E-rosette depletion (E^+ obtained after erythrocyte lysis), and finally treatment with anti-T3 antibody, anti-DR antibody, and complement. Aliquots from each stage of separation were activated in rIL-2 for 3 days at 1,000 U/ml and tested in 4-hour ^{51}Cr-release assay against a fresh tumor cell preparation of a human fibrosarcoma.

b. Lysis expressed in lytic units per 10^6 cells, where one lytic unit is the number of cells required to lyse 30% of 10^4 target cells.

c. Lysis = 59% at minimum effector/target ratio tested (5:1).

tration. Yet when LAK cells were transferred and systemic rIL-2 was administered, the mean number of metastases was reduced by 88% (12).

LAK cells and rIL-2 have been shown to significantly reduce metastases in similar experiments where other syngeneic tumors (including sarcomas, melanomas, and a colon adenocarcinoma) have been used; a total of seven different tumors tested with three distinct histologies showed a reduction in metastases (10, Papa, M. Z., et al., manuscript in preparation).

In addition, a new murine hepatic metastatic model was developed (13) using intrasplenic tumor injection to establish multiple metastases in the liver. Treatment identical to that for pulmonary metastases was administered and again established 3-day-old metastases from a variety of tumors were reduced > 90% by LAK and RIL-2 therapy (Figure 3).

In vitro data on the phenotype of the murine LAK cell precursor demonstrated that it was Thy-1.2-negative and asialo GM1-positive. In order to test this in the in vivo pulmonary sarcoma model, LAK cells were generated from splenocytes depleted of either Thy-1.2-bearing cells or asialo GM1-bearing cells by antibody and C′ and were used to treat mice

with MCA-105 sarcoma pulmonary metastases. In both experiments, intravenous transfers of LAK cells generated from C'-treated splenocytes were able to reduce pulmonary metastases when given with rIL-2. When LAK cells generated from Thy-1.2$^-$ splenocytes were transferred with IL-2, these cells remained capable of mediating regression equal to that of LAK cells from C'-treated splenocytes. Yet when LAK cells generated from splenocytes treated with anti-asialo GM1 and complement was used with rIL-2, no regression of metastases was seen (Table VIII). Therefore, the in vitro characterization of the LAK cell precursor (Thy-1.2-negative and asialo GM1-positive) correlated with in vivo activity.

Mechanisms of In Vivo Lymphokine-activated Killer Cell and Recombinant Interleukin 2 Antitumor Activity

When LAK cell and rIL-2 therapy was shown to mediate tumor regression in vivo, several studies were undertaken to analyze this phenomenon. Initially, serial histologic studies of regressing tumors were performed to identify cells mediating this regression. Three days after intravenous

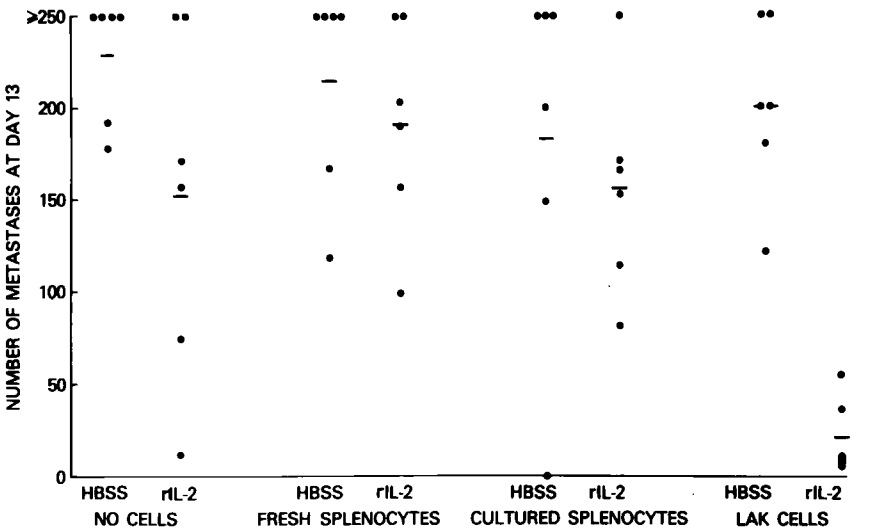

Figure 2. Three days after the intravenous injection of 3 × 10^5 MCA 105 sarcoma cells, mice began therapy with either splenocytes cultured in medium alone, fresh splenocytes, or splenocytes cultured in rIL-2 (LAK cells) either alone or in conjunction with the administration of rIL-2. On days 3 and 6 after tumor injection 10^8 cells were given, and 15,000 U of rIL-2 was administered intraperitoneally every 8 hours from days 3 through 8 after tumor injection. Only mice that received LAK cells plus rIL-2 showed a significant decrease in the incidence of pulmonary metastases ($P < 0.01$).

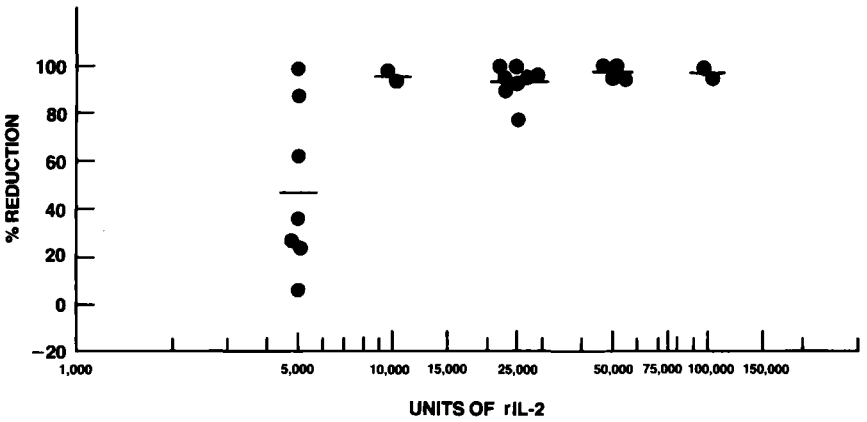

Figure 3. Effect of LAK cells plus increasing doses of rIL-2 on MCA 105 liver metastases in 16 consecutive experiments. Each animals was injected with 3 × 10^5 MCA 105 cells and treated with rIL-2 i.p. every 8 hours starting on day 3. Increasing doses of rIL-2 led to increasing reduction in the number of hepatic metastases when 10^8 LAK cells were administered i.v. concurrently on days 3 and 6. Each point represents the percent reduction in the mean number of metastases of LAK cell and rIL-2-treated mice compared to rIL-2 alone-treated mice in an individual experiment.

Table VIII. In Vivo Efficacy of Lymphokine-activated Killer Cells after Treatment of Lymphokine-activated Killer Precursors with Antibody and Complement

Treatment	*Number of metastases per animal*	*Mean number of metastases*
Experiment 1		
HBSS	81,250,250,250,250,250	222
rIL-2	0,157,160,161,181,250	151
C'-treated LAK and rIL-2	6,6,8,26,32,45	20
Anti-Thy-1.2 + C'-treated LAK and IL-2	0,16,40,97	37
Experiment 2		
HBSS	27,138,250,250,250	183
rIL-2 only	3,149,250,250,250,250	192
C'-treated LAK and rIL-2	1,1,1,3,3,13,15,44	10
Anti-asialo GM1 + C'-treated LAK and rIL-2	39,90,250,250,250,250	188

Adapted from Yang et al., manuscript submitted for publication.

injection of a single-cell suspension of tumor cells, multiple foci are established in the lung and are actively expanding. If LAK cells and rIL-2 are given at this time, the rapid infiltration of the tumor nodules with activated lymphocytes is seen. As time progresses, the lymphocytic infiltrate intensifies and necrosis of tumor cells is seen. Tumor debris is removed by phagocytosis by macrophages that appear later, and only confluent lymphocytes and macrophages remain. This infiltrate then rapidly resolves after termination of treatment with rIL-2. Furthermore, if the lungs of an animal receiving LAK and rIL-2 treatment are enzymatically digested and the infiltrating lymphocytes isolated on a percoll gradient, the yield of lymphocytes is increased two- to five-fold over animals treated only with IL-2, and these lymphocytes exhibit LAK activity in ^{51}Cr-release assay (Table IX).

When normal mice not bearing tumors were given LAK cells and rIL-2, their lungs became infiltrated with activated, proliferating lymphocytes. When low doses of rIL-2 are given alone, this marked interstitial lymphocytosis is not seen, implying that these cells are derived from the adoptively transferred LAK cell population. This was confirmed when studies treating B6-PL/CY-Thy-1 mice with LAK cells from congenic C57BL/6

Table IX. Lytic Activity of Lymphocytes Extracted from Lungs of Animals Receiving Interleukin 2 and Lymphokine-activated Killer Cell Therapy[a]

	Lysis of MCA-102 (Effector/target = 100:1)		*Mean number of cells extracted per lung ($\times 10^{-6}$)*	
Duration of therapy[b]	*IL-2 only*	*LAK + IL-2*	*IL-2*	*LAK + IL-2*
2 days	0 ± 2	18 ± 3	3.5	16.5
3 days	−4 ± 0	34 ± 6	11.0	23.0
5 days	−3 ± 2	24 ± 5	20.0	36.0

Adapted from Mule, et al., (14).

a. Animals bearing MCA 105 pulmonary metastases are treated on day 3 after tumor innoculation with either IL-2 7,500 U i.p. three times a day or IL-2 and an i.v. transfer of 10^8 LAK cells on day 3 and day 6. Mice are sequentially sacrificed, their lungs enzymatically digested and lymphocytes extracted on a percoll gradient. These lymphocytes are then tested for lysis of fresh MCA 102 tumor in 4-hour ^{51}Cr-release assay.

b. Two and three days of therapy follow a single LAK cell transfer and ongoing IL-2 administration whereas 5 days of therapy is after two LAK cell transfers and continued IL-2 therapy.

mice (differing only in expression of Thy-1.2 antigen on T cells of C57BL/6 mice instead of Thy-1.1 as in B6-PL/CY-Thy-1 mice) and rIL-2 demonstrated that the vast majority of infiltrating cells are donor-derived as evaluated by fluorescent anti-Thy 1.2 antibody (15).

In order to investigate further the mechanics of LAK activity in vivo, an assay of lymphocyte infiltration of organs and rIL-2-induced proliferation was devised (15). Mice were given either no cells or intravenous LAK cells followed by varying doses and schedules of rIL-2. At several time points, the in vivo incorporation of ^{125}IUdR by actively dividing cells was assessed in several organs and tissues. The mice were first given 25 μg of FUdR i.p. to deplete endogenous thymidine monophosphate pools and 30 minutes later, 1 μCi of ^{125}IUdR was given. This thymidine analogue is incorporated into the DNA of dividing cells.

Twenty hours later, the mice were killed, and organs and tissues were removed, weighed, and counted for incorporation of radioactive IUdR. The ratio of IUdR uptake by the tissues of treated mice to the uptake of those same tissues in untreated mice was termed the proliferation index (PI). This index reflects both cell traffic to a specific site and the proliferation rate (DNA synthetic rate) of cells at that site.

When mice were given low doses of rIL-2 with either fresh or cultured splenocytes, only small changes in IUdR uptake were seen when compared to untreated mice (Figure 4). As previously shown, when these treatments are administered to mice bearing pulmonary metastases, no reduction in metastases is seen. In contrast, when LAK cells and rIL-2 are given to mice and IUdR uptake is measured, incorporation is increased sixfold in the lung and in vivo tumor regression can be demonstrated with this regimen. When rIL-2 was given at a constant daily dose, either once a day or in two and three divided doses in conjunction with LAK cells, a maximum proliferation index was obtained with three divided doses of rIL-2 and this dose schedule was associated with the largest in vivo antitumor effect. This supports the hypothesis that in vivo proliferation of adoptively transferred LAK cells in the tumor bearing host is required for tumor regression and that increased proliferation is associated with increased therapeutic benefit.

Our studies have suggested that the maximal benefit of immunotherapy is attained when the greatest number of LAK cells and largest tolerated doses of IL-2 are given. Mule et al. (16) treated mice bearing 3-day-old pulmonary metastases with five consecutive daily transfers of 10^8 LAK cells per day accompanied by 25,000 U of rIL-2 every 8 hours for 5 days and demonstrated a prolonged mean survival of 86 $\pm$ 11 days vs. 46 $\pm$ 2 days for treatment with HBSS or 57 $\pm$ 4 days for rIL-2 alone. Twenty-

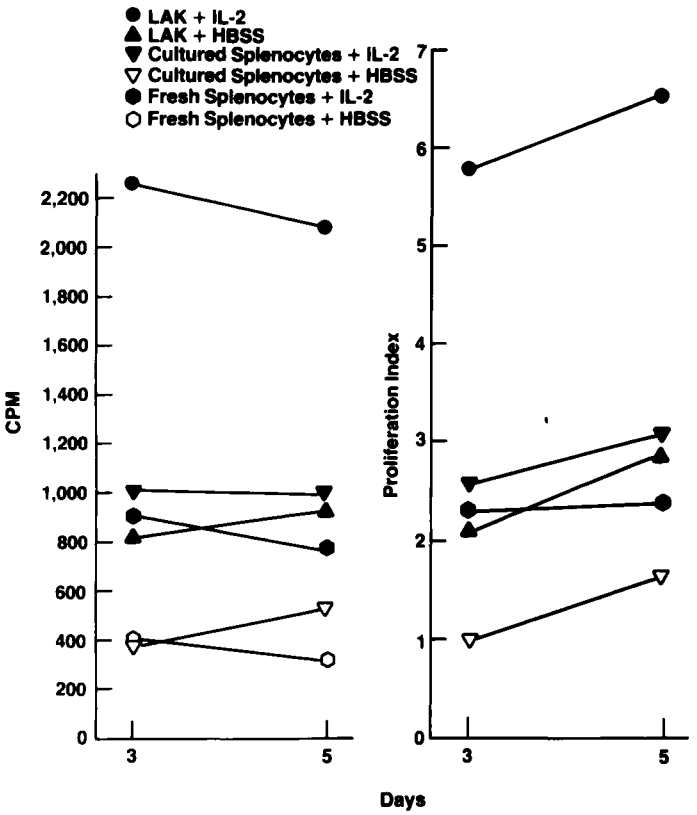

Figure 4. In vivo lymphoid proliferation in the lungs of IL-2-treated mice receiving LAK cells and cultured and fresh splenocytes. Lymphokine-activated killer cells and cultured and fresh splenocytes (8.6×10^7 cells) were injected i.v. in HBSS on day 0 into recipient mice. Each group then received IL-2 6,000 U or HBSS i.p. three times a day from day 0 through 5. On day 2 and 4, three mice per group were given FUdR (25 μg) and ^{125}IUdR (1 μCi) i.p. Twenty hours later, their lungs were harvested and were counted. The mean cpm (*left panel*) and PI (*right panel*) (mean cpm of experimental group divided by mean cpm of lungs from mice receiving fresh splenocytes plus HBSS) were then calculated and are plotted.

five percent of mice receiving LAK and rIL-2 treatment showed survival out to 140 days, whereas no HBSS-treated mouse lived more than 53 days. Treated animals that show prolonged survival, but eventually die of tumor, show only one or two large residual deposits of tumor in contrast to untreated animals that typically die with hundreds of pulmonary metastases. These deposits of tumor that escape LAK and rIL-2 therapy have been examined and remain susceptable to in vitro lysis by LAK and new pulmonary metastases generated from these deposits in fresh

hosts are responsive to LAK and rIL-2 treatment. The explanation for this "escape" of therapy is unknown.

Considerations for Therapy of Human Tumors with Lymphokine-activated Killer and Recombinant Interleukin 2

The extensive experience with LAK cells and IL-2 therapy in murine tumors has led to certain guidelines and objectives in the treatment of patients. It would appear that the maximal benefit is obtained when LAK cell and rIL-2 therapy are given simultaneously. Dose titration studies in the mouse, predict that, on a proportionate weight basis, up to 3×10^{11} LAK cells may be required for successful therapy. Previous immunosuppression of the tumor bearing host (e.g., radiation or chemotherapy) does not abrogate the effectiveness of LAK cell and rIL-2 therapy in mice. This is in contrast to many other forms of immunotherapy that require an immunologically intact tumor-bearing host (often not the case in patients receiving other therapy). This may be of great value in multimodality therapy schemes.

With regards to the applicability of LAK cells and rIL-2 therapy to humans, several preliminary studies have been performed. It has previously been shown (17) that IL-2 can safely be given to patients in doses up to 2 mg (6×10^6 U) as a bolus or constant infusion. More recently, patients have received up to 100,000 U/kg every 8 hours by intermittent infusion with significant but ameliorable toxicity.

Our previous experience with the acquisition, activation, and reinfusion of large numbers of human peripheral blood lymphocytes has been described (18). Between 1.6 and 17.1×10^{10} phytohemagglutinin-stimulated lymphocytes were reinfused into patients and this was well tolerated and all accomplished in the outpatient setting.

At the present time, clinical trials of LAK cells and rIL-2 therapy are underway in patients with metastatic cancer who have failed all conventional therapy (19). Further follow-up on these patients will be necessary to determine the benefits of IL-2 and LAK cell-adoptive therapy in humans.

Summary

Lymphokine-activated killer cells are generated by the incubation of lymphocytes in IL-2 for 3–5 days. Lymphokine-activated killer cells demonstrate the ability to lyse fresh noncultured tumor cells in short-term ^{51}Cr-release assays without lysing normal tissues. They are generated from the null cell population, devoid of B-cell or T-cell markers, and share

the asialo GM1 marker with NK cells. After IL-2 activation, they express some T cell markers.

In adoptive cell transfer experiments treating established growing tumors, LAK cells administered with IL-2 can mediate the marked regression of murine tumor metastases from a variety of tumor types. This in vivo effect is mediated by the infiltration of tumor nodules with activated lymphocytes demonstrating LAK activity in vitro. This lymphocytic infiltration is associated with active proliferation, and when this proliferation is enhanced, increasing therapeutic benefits are obtained. The adoptive transfer of LAK cells plus IL-2 is now being applied to the treatment of patients with metastatic cancer.

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Molecular Analysis of Interferon-Gamma and Lymphotoxin

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Introduction

Lymphokines are soluble factors secreted from lymphocytes which are important in the activation of cell-mediated immunity. This chapter will describe the cloning of DNAs for interferon-gamma (IFNγ) and lymphotoxin (LT), two lymphokines which have numerous biologic activities.

Interferon-gamma was first identified by its potent antiviral activity (1) but was shown to be distinct from the virally induced IFNα and IFNβ by its sensitivity to pH 2 treatment. Interferon-gamma was previously termed immune IFN because it is produced by antigen-stimulated T lymphocytes (2), or type II IFN because IFNγ is antigenically and biochemically distinct from the type I IFNs induced by viruses (3).

Interferon-beta and the IFNα gene family share structural homology and are closely linked on chromosome 9 in humans (reviewed in Weissmann and Weber [4]). In addition, IFNα and IFNβ are recognized by the same cell surface receptor, which is distinct from the IFNγ receptor (5). The biologic activities of IFNγ are also different from the type I IFNs, and IFNγ appears to have greater immunomodulatory properties (6).

The absolute determination of the physical and biologic properties of IFNγ were impeded by the lack of a pure and abundant source. Once the complementary DNA (cDNA) for IFNγ was isolated and expressed in heterologous systems, recombinant IFNγ became available for biologic testing. The importance of this lymphokine in the regulation of the immune system has subsequently been confirmed.

Isolation of Interferon-Gamma Complementary DNA Clones

The cDNA for human IFNγ was first isolated in 1981 by the group at Genentech (7). Human peripheral blood lymphocytes were stimulated for 24 hours with mitogens and then used for the preparation of messenger RNA (mRNA). The mRNA was fractionated on acid urea-agarose gels, injected into *Xenopus* oocytes, and subsequently assayed for antiviral activity. Active fractions were approximately 18S in size and were enriched

approximately 20-fold. The active mRNA fraction was used to prepare a cDNA library by standard techniques in the *PstI* site of pBR322. Duplicate copies of 8,300 clones were prepared on nitrocellulose filters and screened with two probes. The "induced probe" was radiolabeled cDNA prepared from 18S mRNA of an induced culture and the "uninduced probe" was radiolabeled cDNA prepared from 18S mRNA of an uninduced culture (which contained no antiviral activity in the culture supernatant).

The great majority of clones had similar hybridization profiles with both probes. However, about 1% of the cDNA clones hybridized weakly with the induced probe, but undetectably with the uninduced probe. These "induced clones" were further characterized by restriction endonuclease analysis and cross-hybridization studies. Surprisingly, all of the "induced clones" appeared to be related. The clone containing the plasmid with the longest cDNA insert was chosen for DNA sequencing analysis. The amino acid sequence encoded by the cDNA is presented in Figure 1.

The amino-terminus of the encoded protein had several features in common with typical eukaryotic signal sequences: the initial methionine is followed by a basic residue and then 18 amino acids which are predominantly hydrophobic. Sequence identity with IFNα was observed at positions 18 to 21 (ser-leu-gly-CYS), where cysteine was the first residue of secreted mature IFNα. Consequently, the first cysteine was assumed to be the amino terminus of mature IFNγ and was chosen for engineered expression in *Escherichia coli*. Synthetic DNA was utilized to join the coding sequence to an expression plasmid containing the *E. coli trp* promoter.

The cDNA was also engineered for direct expression in yeast (8) and for secretion in mammalian cells (7). All three expression systems produced an antiviral activity that was biochemically and antigenically characteristic of IFNγ. The activity was species specific and sensitive to pH 2 and 0.1% sodium dodecyl sulfate (SDS). Antibodies prepared against natural IFNγ neutralized all of the recombinant IFNγ antiviral activity. These studies confirm the isolation and expression of human IFNγ cDNA.

The IFNγ cDNA was used as a probe to identify the human IFNγ gene in a human genomic λ-recombinant DNA library (9, 10). The IFNγ gene is encoded by a single gene containing three intervening sequences, as shown in Figure 2. This is in contrast to the IFNα gene family and the IFNβ gene which contain no intervening sequences. The IFNγ gene is encoded by chromosome 12 in humans (11, 12), whereas IFNα and IFNβ genes are on chromosome 9 (12, 13). There is no apparent DNA

HUMAN INTERFERON-GAMMA

```
-23                                                                  met lys tyr
-20  thr ser tyr ile leu ala phe gln leu cys ile val leu gly ser leu gly cys tyr cys
  1  GLN ASP PRO TYR VAL LYS GLU ALA GLU ASN LEU LYS LYS TYR PHE ASN ALA GLY HIS SER  20
     ASP VAL ALA ASP ASN GLY THR LEU PHE LEU GLY ILE LEU LYS ASN TRP LYS GLU GLU SER  40
     ASP ARG LYS ILE MET GLN SER GLN ILE VAL SER PHE TYR PHE LYS LEU PHE LYS ASN PHE  60
     LYS ASP ASP GLN SER ILE GLN LYS SER VAL GLU THR ILE LYS GLU ASP MET ASN VAL LYS  80
     PHE PHE ASN SER ASN LYS LYS LYS ARG ASP ASP PHE GLU LYS LEU THR ASN TYR SER VAL 100
     THR ASP LEU ASN VAL GLN ARG LYS ALA ILE HIS GLU LEU ILE GLN VAL MET ALA GLU LEU 120
     SER PRO ALA ALA LYS THR GLY LYS ARG LYS ARG SER GLN MET LEU PHE ARG GLY ARG ARG 140
     ALA SER GLN                                                                     143
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Figure 1. Protein sequence encoded by IFNγ cDNA. The first 23 residues serve as a signal sequence. In 1984 Rinderknecht et al. (17) determined the amino-terminal sequence of natural mature IFNγ and that the carboxy-terminal 10 residues are removed.

homology and little (if any) protein homology between IFNγ and IFNα or IFNβ.

Interferon-Gamma Protein Structure

The characterization of IFNγ cDNA aided the understanding of the IFNγ protein structure. Reports prior to 1982 described the size of IFNγ as 40,000–80,000 mol wt, while cDNA cloning suggested a (nonglycosylated) size of 17,000 mol wt. Yip et al. (14) demonstrated that natural IFNγ could be resolved into two monomeric bands of 20,000 and 25,000 mol wt on SDS-polyacrylamide gels; these results suggested that earlier size estimates may have been due to aggregation. Interferon-gamma readily aggregates to form dimers, which is probably the biologically active form (15, 16).

Natural IFNγ is heterogeneous in both size and charge. The majority of this variability is caused by differential glycosylation. Two potential *N*-linked glycosylation sites are found in the IFNγ sequence. Both sites are glycosylated in the 25,000-mol wt form, although the 20,000-mol wt IFNγ contains carbohydrate at only the first site, asparagine 25 (17). A small amount of nonglycosylated natural IFNγ has also been observed (18).

Rinderknecht et al. (17) demonstrated that natural human IFNγ differed from the structure predicted by the cDNA. The amino-terminus

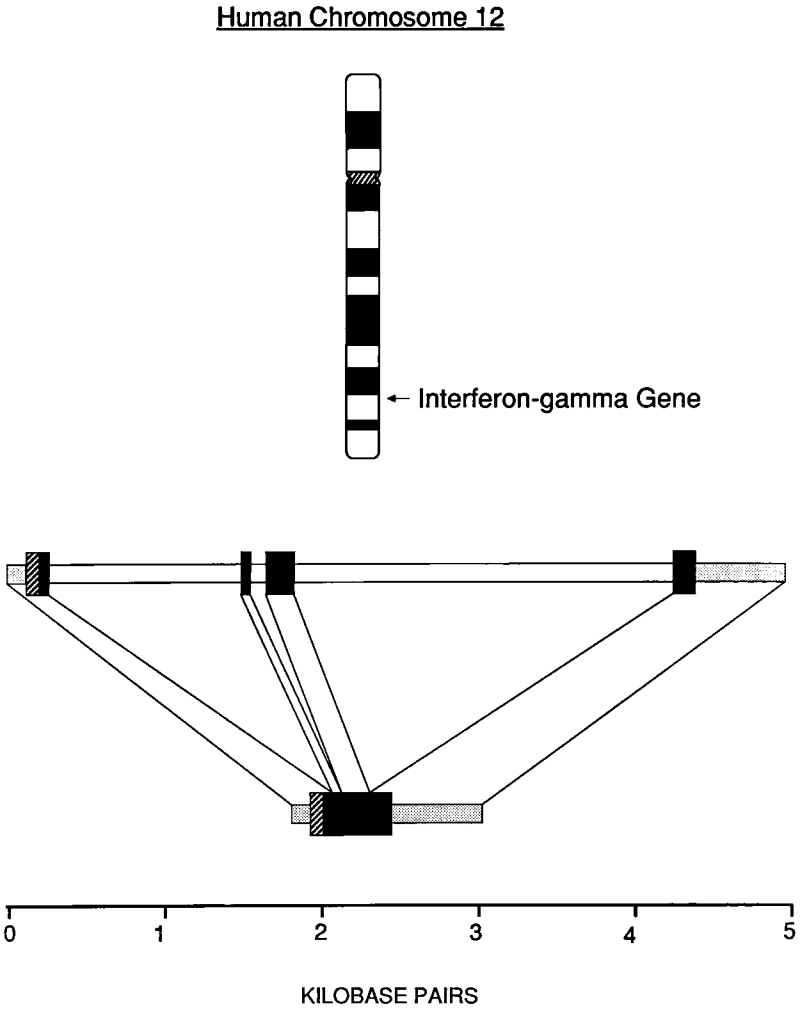


Figure 2. Gene structure of IFNγ. Human IFNγ is encoded on the long arm of chromosome 12 (see Naylor et al. [11], Trent et al. [12]). The IFNγ gene contains three introns and a similar arrangement is observed in human, bovine, and murine DNA (see Gray and Goeddel [25]). The stippled boxes represent 5′ (*left*) and 3′ (*bottom*) untranslated sequences; the hatched box represents the signal coding region; the filled boxes represent mature coding sequence; and the open boxes are introns.

of natural IFNγ is pyroglutamate, in contrast to cysteine as predicted by weak homology with human IFNα (7). This result demonstrated that the signal sequence for IFNγ is 23 rather than 20 residues in length. In addition, the first amino acid of secreted IFNγ is glutamate, which sub-

sequently cyclizes to the pyroglutamate derivative. The carboxy-terminus of natural IFNγ is quite variable and six different termini have been observed (17). Deletion of 12 carboxy-terminal residues has no effect on biologic activity. However, removal of 18 residues (by recombinant methods) reduces the specific activity to only 1% (19).

A specific cell surface receptor for IFNγ has been identified. Radiolabeled recombinant IFNγ binds to monocytes or cell lines in a saturable and reversible manner (20–23) with an affinity of 1–5 $\times$ 10^8 M^{-1} and approximately 3,000–7,000 binding sites per cell. This binding can be inhibited by unlabeled IFNγ but not by IFNα or IFNβ. Cross-linking experiments with iodinated IFNγ suggest that the size of the receptor is approximately 70,000 mol wt (21, 23).

Animal Interferon-Gamma Sequences

Human IFNγ is not active in the tumor model systems of the mouse. Consequently, the murine IFNγ gene was isolated by screening a murine genomic λ library with the human cDNA sequence (24). The murine IFNγ gene structure is quite similar to the human gene with three intervening sequences of similar lengths in homologous positions. The overall DNA homology is 65% (25) and the murine gene has been localized to chromosome 10 (26), which has several gene loci homologous to human chromosome 12. The murine IFNγ protein is only 40% homologous with human IFNγ and is 10 residues shorter at the carboxy-terminus. Natural murine IFNγ is a glycoprotein with an apparent molecular weight of 20,500 on SDS-polyacrylamide gels and 41,000 on sieving columns (27).

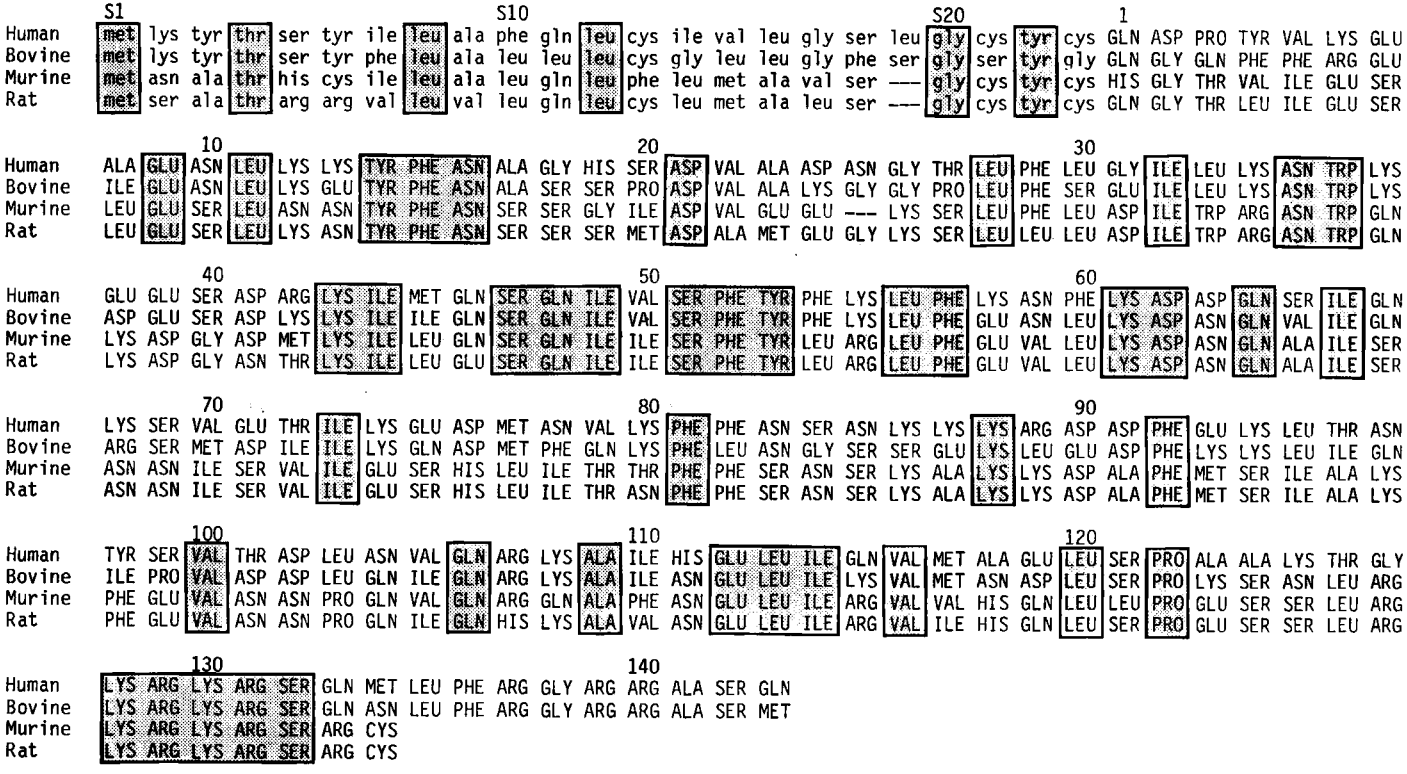
Sequences for rat IFNγ (28) and bovine IFNγ (29) have also been reported. Homology comparisons of these four IFNγ amino acid sequences are presented in Figure 3. Interferon-gamma antiviral activity is species specific, which is probably due to the low homology observed between species. Only closely related organisms, such as rat and mouse, have IFNγ sequences that are closely related (87%) and activities that cross react (28).

Interferon-Gamma Biologic Activities

The characterization of IFNγ cDNA and its expression in heterologous systems has provided recombinant IFNγ for biologic studies. This pure reagent has been tested by many investigators in numerous biologic systems. Interferon-gamma clearly exhibits an array of activities in vitro and in vivo.

Interferon-gamma was originally identified by its antiviral activity (1) and recombinant murine IFNγ has recently been shown to protect mice

Interferon-Gamma Protein Homology



in vivo from a lethal challenge with encephalomyocarditis virus (29). Interferon-gamma exhibits an antiproliferative activity on numerous cell lines in vitro (29, 30), and its potential role as an antitumor agent is currently under investigation in human clinical trials.

Interferon-gamma is an important immunomodulatory agent. It induces both class I and class II antigens of the major histocompatibility complex (31–33). Class II expression is necessary for antigen presentation by macrophages and may be responsible for the enhancement of antibody production by IFNγ in vivo (34). It may also stimulate resting B cells to secrete antibody in vitro (35) and appears to promote B cell differentiation (36).

Resting macrophages require a signal to become cytotoxic toward tumor cells. Recombinant IFNγ has been shown to be a potent macrophage activation factor by several groups (37–40). Interferon-gamma appears to be the predominant activation factor found in activated lymphocyte supernatants, in that antibodies to recombinant IFNγ neutralize this activity (41). Interferon-gamma is necessary in vivo for the clearance of *Leishmania* infections; mice receiving antibodies to IFNγ did not effectively clear the infection, whereas control mice did (42).

Interferon-gamma regulates the activity of other lymphokines by inducing their expression and by enhancing their action: IFNγ induces macrophages to secrete the cytokine interleukin 1 (43), which is important in T cell growth, antigen presentation, and inflammation. Interferon-gamma also enhances the production of interleukin 2 (T-cell growth factor) and lymphotoxin by lymphocyte cultures (44). Interferon-gamma acts synergistically with other lymphokines: antiviral and antiproliferative activities of IFNα or IFNβ are enhanced five- to tenfold in the presence of IFNγ (45); IFNγ also enhances tumor cell lysis with lymphotoxin or tumor necrosis factor (46, 47).

Lymphotoxin Complementary DNA Cloning

Lymphotoxin was initially identified by its anticellular activity on several tumor cell lines (48–50): LT exhibits cytolytic activity on some neoplastic cell lines and cytostatic activity on others. Primary cell cultures and some tumor lines are not affected by LT. Early in vivo studies with partially purified LT suggested that LT has an antitumor effect (51–53).

Figure 3. Homology of human, bovine, murine, and rat IFNγ. The residues in common are boxed. The human sequence shares 61%, 40%, and 39% homology with bovine, murine, and rat IFNγ, respectively. Bovine IFNγ shares 44% and 39% homology with the murine and rat sequences.

Aggarwal and colleagues reported the first convincing purification of LT to homogeneity (54, 55). Lymphotoxin was isolated from culture supernatants of the human lymphoblastoid cell line RPMI-1788 by adsorption to controlled pore glass, DEAE-cellulose chromatography, lentil lectin sepharose chromatography, and preparative native polyacrylamide gel electrophoresis. The purified LT has a monomer size of 25,000 mol wt and chromatographs on sieving columns with a size of 65,000 mol wt. Homogeneous LT was subjected to microsequence analysis and 155 residues were determined (55). A small number of carboxy-terminal residues remained unidentified because of the limited availability of material.

A synthetic gene was constructed by the group at Genentech which encoded the 155 residues determined by sequencing, preceded by an ATG translational initiation codon (56). This synthetic gene design assumed that the unknown carboxy-terminal residues would not be necessary for activity, as has been observed for some other proteins such as IFNγ (19). The LT synthetic gene was constructed in three segments from groups of synthetic oligonucleotides 16–20 bases in length (56, 57). These segments were individually cloned and sequenced, and then ligated together with an expression plasmid containing a *trp* promoter. Cultures of *E. coli* containing this expression plasmid were prepared, but extracts were inactive when assayed for cytolysis of murine L-929 tumor cells. This result suggested that the carboxy-terminal residues not identified by protein sequencing (and consequently not encoded by the synthetic gene) were necessary for LT activity.

The synthetic LT gene was used as a probe to identify a natural LT cDNA sequence (56). Messenger RNA was isolated from a culture of stimulated human peripheral blood lymphocytes and utilized for the preparation of cDNA. This cDNA was cloned in the vector λgt10. The resulting library was screened with radiolabeled synthetic gene segments under conditions of low stringency. Several phage hybridized with all three segments and the longest cDNA insert was sequenced. The longest open reading frame of this cDNA predicted a sequence which was completely homologous with the sequence determined by protein sequencing (presented in Figure 4). Preceding the mature LT sequence are 34 residues with characteristics of a signal sequence (58). The cDNA also encoded 16 additional residues at the carboxy-terminus which were not identified by protein sequencing. Restriction endonuclease sites common to both natural and synthetic genes were utilized to splice DNA encoding the additional 16 residues into the expression plasmid. Cultures containing the hybrid synthetic gene/natural cDNA expression plasmid produced

```
-34                          met thr pro pro glu arg leu phe leu pro arg val cys gly
-20  thr thr leu his leu leu leu leu gly leu leu leu val leu leu pro gly ala gln gly
  1  LEU PRO GLY VAL GLY LEU THR PRO SER ALA ALA GLN THR ALA ARG GLN HIS PRO LYS MET  20
     HIS LEU ALA HIS SER THR LEU LYS PRO ALA ALA HIS LEU ILE GLY ASP PRO SER LYS GLN  40
     ASN SER LEU LEU TRP ARG ALA ASN THR ASP ARG ALA PHE LEU GLN ASP GLY PHE SER LEU  60
     SER ASN ASN SER LEU LEU VAL PRO THR SER GLY ILE TYR PHE VAL TYR SER GLN VAL VAL  80
     PHE SER GLY LYS ALA TYR SER PRO LYS ALA THR SER SER PRO LEU TYR LEU ALA HIS GLU 100
     VAL GLN LEU PHE SER SER GLN TYR PRO PHE HIS VAL PRO LEU LEU SER SER GLN LYS MET 120
     VAL TYR PRO GLY LEU GLN GLU PRO TRP LEU HIS SER MET TYR HIS GLY ALA ALA PHE GLN 140
     LEU THR GLN GLY ASP GLN LEU SER THR HIS THR ASP GLY ILE PRO HIS LEU VAL LEU SER 160
     PRO SER THR VAL PHE PHE GLY ALA PHE ALA LEU                                    171
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Figure 4. Protein sequence encoded by human LT cDNA. The first 34 residues serve as a signal sequence. Natural LT is glycosylated at position 62.

cytolytic activity when assayed on L-929 cells. This activity could be neutralized by antibodies prepared against natural LT (56).

Lymphotoxin Protein Characteristics

The cloning and expression of LT in bacteria should provide sufficient recombinant LT for biologic and physicochemical studies. A murine monoclonal antibody to natural human LT has been isolated and utilized for immunoaffinity purification (56). Nearly homogeneous recombinant LT can be isolated from *E. coli* cultures by ammonium sulfate precipitation followed by chromatography on the monoclonal antibody conjugated column.

Natural LT is glycosylated at position 62 and consequently exhibits a higher molecular weight than the nonglycosylated recombinant LT (25,000 compared with 18,600). Other biochemical parameters of recombinant and natural LT are very similar. Both preparations tend to aggregate under nondenaturing conditions and chromatograph as multimers on molecular sieving columns. Natural and recombinant LT both have similar thermolability profiles with T ½ of 70°C (57). Both preparations are relatively insensitive to the proteases trypsin, chymotrypsin, *Staphylococcus aureus* V8 protease, thermolysin, and lysine C peptidase (59).

The carboxy-terminal region of LT is composed of generally hydrophobic residues and is probably buried in the interior of the molecule. This is consistent with an observed resistance to carboxypeptidase

treatment. The amino-terminal region of LT is relatively hydrophilic and susceptible to proteolysis. Two forms of natural LT have been isolated from RPMI-1788 cells which differ in their amino termini (54); the smaller form (20,000 mol wt) lacks 23 residues and is presumably a degradation product of the larger form. Both of these LT forms have been expressed in *E. coli* and have similar specific activities (57).

The cytolysis of tumor cells caused by LT appears to be mediated by a cell surface receptor (60). Radiolabeled human LT (both natural and recombinant) was fully active in cell lysis and bound with high affinity to a single class of murine L-929 receptors ($K_d - 6.7 \times 10^{11}$ M). Binding was specific and could be inhibited with unlabeled LT or LT antibodies. L-929 cells contained approximately 3,200 binding sites per cell.

The only protein that exhibits significant sequence homology to LT is tumor necrosis factor (TNF), which was purified (61) and the cDNA structure was elucidated (62) by the group at Genentech. The mature forms of LT and TNF share 35% amino acid homology and contain additional conservative amino acid changes (61). This homology is reflected in hydrophilicity plots, which are similar for both proteins (63). In addition, LT and TNF exhibit very similar biological activities.

Lymphotoxin Gene Structure

The human LT gene was isolated from a human genomic λ-recombinant library utilizing the synthetic gene segments as probes (64). The LT gene contains three introns and is approximately two kilobase pairs in length, as shown in Figure 5. The first intron is 287 base pairs (bp) in length and interrupts the 5′-untranslated region. The second and third introns are 86 and 247 bp and interrupt the signal and mature coding sequences, respectively. The LT transcript is 16S in size (56) and is preceded by a characteristic "TATA" box (TATAAA) 28 bp upstream of the putative cap site (64).

The TNF gene also contains three intervening sequences and both genes are closely linked on human chromosome 6 (64). Surprisingly, only 1,220 bp separates the 3′ end of the LT gene from the 5′ end of the TNF transcription start (65). Whereas the 5′ ends of these genes are not related, the last exons are significantly homologous (56% at the DNA level). The last exon of each gene codes for more than 80% of the secreted proteins; consequently, the mature proteins are quite homologous.

Although the LT and TNF genes are very closely linked, their regulation is quite distinct. Tumor necrosis factor is produced by macrophages 2–24 hours after induction, whereas LT is produced by lymphocytes 8–72 hours after induction (65). Little homology is observed between the puta-

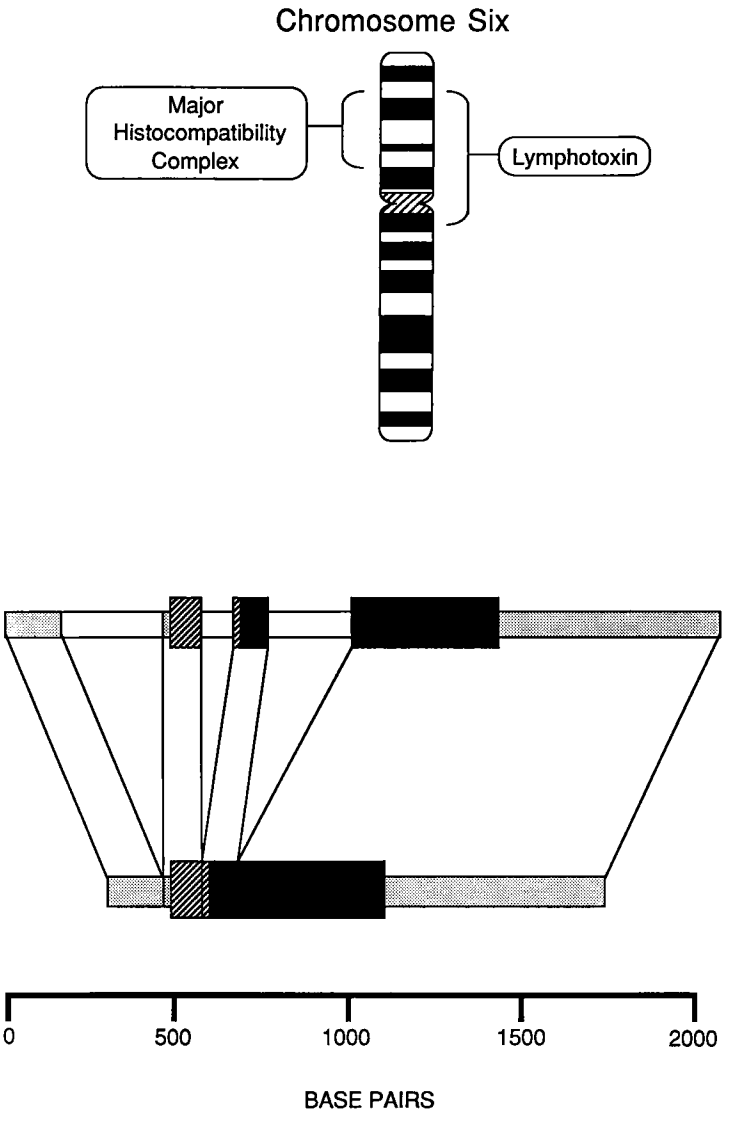

Figure 5. Structure of the human LT gene. The LT gene maps on human chromosome 6 near the region coding for the major histocompatibility complex. The primary transcript contains three intervening sequences which are excised in the mRNA (*bottom*).

tive promoter regions of the LT and TNF genes (64), which is consistent with their independent regulation.

Lymphotoxin Biologic Activity

Lymphotoxin was initially characterized by its in vitro anticellular activity. Lymphotoxin causes the rapid lysis of some tumor cell lines and exhibits antiproliferative activity on others (66, 67). Some tumor cell lines are not growth inhibited by LT, similar to normal cell lines and primary cell cultures.

Lymphotoxin exhibits a direct antitumor effect in vivo. In the classic tumor necrosis assay (68), LT causes the rapid hemorrhagic necrosis of methylcholanthrene-induced sarcoma in susceptible mice (56). This effect is observable within 24 hours; some of the treated mice clear the tumor after prolonged treatment.

Lymphotoxin may be an important regulatory molecule of the immune system: LT stimulates granulocytes in an antibody-dependent cellular cytotoxicity assay (69, 70): LT also activates osteoclasts to resorb bone tissue in vitro (71); LT and TNF exhibit a potent synergistic anticellular activity in vitro (46, 47). This synergy may be a result of an increase of LT receptors induced by IFNγ (72). The potent antitumor activity observed in natural preparations of lymphokines may be a result of the synergistic activity of small amounts of LT and IFNγ (46).

Tumor necrosis factor and LT share the same biologic activities which have been observed to date. This is most likely a result of their recognition by the same receptor (72); LT and TNF may complement each other by exhibiting similar bioactivities but independent modes of regulation.

The cloning and expression of the cDNAs for LT and IFNγ have been instrumental in understanding the biologic actions of these lymphokines. The availability of recombinant-derived proteins will aid in defining their respective roles in the immune system and their potential in controlling neoplasia.

Summary

This manuscript reviews the biochemical characteristics and molecular biology of interferon-gamma and lymphotoxin, two lymphokines which are secreted by activated T lymphocytes. Cloning and expression of the IFNγ cDNA demonstrated a monomer molecular weight of 17,000 for the secreted protein. The active form isolated from sera is a glycosylated dimer of 50,000 mol wt. Interferon-gamma is encoded by a single gene on human chromosome 12. Murine, rat, and bovine genes have similar structures with three introns. Interferon-gamma is recognized by a specific

cell surface receptor, a protein of 70,000 apparent mol wt. Lymphotoxin causes cytolysis of numerous tumor cell lines in vitro. Natural human LT is 25,000 mol wt and its cDNA has been cloned and expressed. Lymphotoxin is encoded by a single gene with three introns on human chromosome 6. The LT gene is closely linked to the gene for tumor necrosis factor and the proteins share 35% amino acid homology. The availability of recombinant lymphokines has aided the biologic characterization of their activities.

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Index of Key Words*

* Page numbers refer only to the first page of the chapter in which the subject is covered and not to the specific occurrence of a particular word.