Annual Review of Immunology Volume 4 1986

Regulation of the Assembly and Expression of Variable-Region Genes
G D Yancopoulos, and F W Alt. Vol. 4: 339–368PDF (693K)

Current Status of Immunological Adjuvants
H S Warren, F R Vogel, and L A Chedid. Vol. 4: 369–388PDF (818K)

Immunoglobulin A: Strategic Defense Initiative at the Mucosal Surface
B J Underdown, and J M Schiff. Vol. 4: 389–417PDF (851K)

The Receptor with High Affinity for Immunoglobulin E
H Metzger, G Alcaraz, R Hohman, J P Kinet, V Pribluda, and R Quarto. Vol. 4: 419–470PDF (1054K)

Murine Major Histocompatibility Complex Class-I Mutants: Molecular Analysis and Structure-Function Implications
S G Nathenson, J Geliebter, G M Pfaffenbach, and R A Zeff. Vol. 4: 471–502PDF (909K)

The Membrane Attack Complex of Complement
H J Muller-Eberhard. Vol. 4: 503–528PDF (652K)

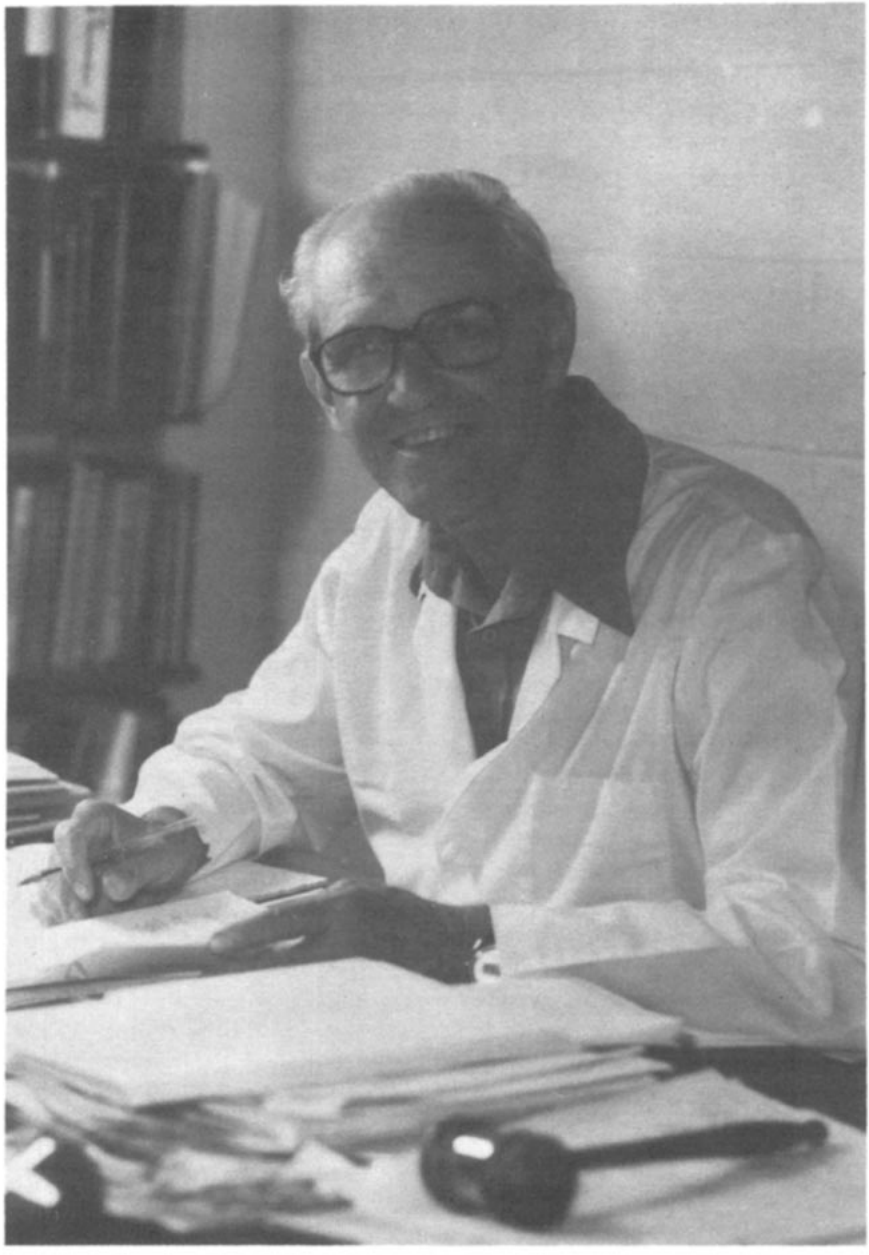
The Molecular Genetics of the T-Cell Antigen Receptor and T-Cell Antigen Recognition
M Kronenberg, G Siu, L E Hood, and N Shastri. Vol. 4: 529–591PDF (1774K)

The Role of the T3/Antigen Receptor Complex in T-Cell Activation
A Weiss, J Imboden, K Hardy, B Manger, C Terhorst, and J Stobo. Vol. 4: 593–619PDF (751K)

The Physiology of B Cells as Studied with Tumor Models
J M Hanley-Hyde, and R G Lynch. Vol. 4: 621–649PDF (865K)

Mechanism of Cytotoxicity by Natural Killer (NK) Cells
R B Herberman, C W Reynolds, and J R Ortaldo. Vol. 4: 651–680PDF (869K)

Cancer Immunotherapy Using Interleukin-2 and Interleukin-2-Activated Lymphocytes
S A Rosenberg, and M T Lotze. Vol. 4: 681–709



David W. Talmage

Ann. Rev. Immunol. 1986. 4 : 1–11

THE ACCEPTANCE AND REJECTION OF IMMUNOLOGICAL CONCEPTS

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INTRODUCTION

Immunology is rich in concepts. Some of these, like "suppressor cells" and "helper cells," are difficult for the outsider to grasp and still more difficult to apply, but others, like "monoclonal antibodies," have been widely applied outside immunology. Still other concepts—e.g. DNA rearrangement—have pioneered scientific advance in a wide area of molecular biology.

The history of immunology is fascinating in part because the central concepts that power its research have changed so rapidly. In the 30 years before 1948, the word "lymphocyte" did not appear in the index of the *Journal of Immunology* (1). Today, half of the papers in that much expanded journal involve research on some aspect of lymphocytes.

What accounts for the rise and fall of biological concepts? It is my thesis that acceptance or rejection of a particular concept by the scientific community does not rest entirely on direct experimental evidence, but on the general milieu of related concepts that have preceded and followed it. This rather simplified version of Kuhn's paradigm theory (2) is based entirely on my own experience during nearly 40 years of research in immunology. It is by no means a documented study of the sociology of scientific advance.

The acceptance of a successful scientific concept appears to go through three distinct phases:

1. General skepticism about a new proposal that runs counter to the general tenets of the day.

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2. Wide awareness of an interesting but controversial hypothesis.
3. General acceptance of the concept as the preferred basis of research in the field.

The emergence of the new concept requires little evidence or support, but instead only an independent mind dissatisfied with current theories. The concept arrives at the second phase when several preparatory concepts that make it plausible have already reached general acceptance. The third phase is reached when the new concept has given birth to numerous successful descendants, i.e. concepts that have made it at least to phase 2.

The above thesis ascribes little credit to direct experimental evidence in the acceptance of a new concept. This is because experimental evidence in biology is rarely universally compelling. Evidence that appears to convince one scientist is frequently discounted by another. Thus, the wide acceptance of a particular concept depends more on the total framework of accepted and acceptable theories than on the experimental evidence designed to test that particular concept.

I will illustrate the above thesis with a description of the origin and acceptance of the cell selection theory, a concept that has interested me for thirty years. I will then attempt to apply the thesis to two of the concepts that are most controversial in immunology today.

CELL SELECTION THEORY

It was 1900 when Paul Ehrlich (3) first introduced a selective theory of immunity before the Royal Society in London. The side-chain theory, as Ehrlich called it, was a remarkable synthesis of the two existing and hotly debated theories of cellular and humoral immunity. Ehrlich had an excellent concept of the interaction between antigen and antibody molecules, cellular receptors and antibody molecules. He also proposed that antibodies were natural body proteins with which antigens made an accidental good fit. The side-chain theory should have launched the beginnings of cellular immunology. Instead it was rapidly forgotten. Like Mendel, Ehrlich was ahead of his time. To the scientists of his day, the side-chain theory seemed so ridiculous that it was not worth considering. His theory never made it past the first phase. When Landsteiner (4) showed that antibodies could be made to newly synthesized chemicals, the side-chain theory and the study of cells were dropped from the consideration of immunologists for more than 30 years.

This was the period of serology (1910–1940). The word "immunology" was invented in 1911 (5), the American Association of Immunologists

founded in 1913 (1), and the *Journal of Immunology* began publication in 1916. Immunologists of this period were primarily concerned with serum antibody. Landsteiner, Heidelberger, and Kabat were the dominant scientists in the field. In 1930, Breinl & Haurowitz (6) published their template theory of antibody formation, which was made more explicit by Pauling (7) in 1940.

Although people paid scant attention to the role of cells in antibody formation (Where did they think antibodies came from?), important progress was made in understanding the basis of immunological specificity. Landsteiner came very close to our modern concept in his discussion of Malkoff's findings (8). Malkoff had come to the conclusion that normal serum contained as many different specific agglutinins as there were sorts of cells that could be agglutinated. Malkoff demonstrated that unabsorbed goat serum could agglutinate pigeon, rabbit, and human blood. He showed, through a process of absorption, that the agglutination of each blood type had to be due to a different antibody. It was possible to absorb out the agglutinins for any two of the blood types and yet find that the third agglutinin remained active.

Because of the large number of different substances that could be agglutinated by normal serum and because each type of agglutinin amounted to several micrograms per milliliter of serum, Landsteiner rejected Malkoff's explanation (4). He was able to purify the agglutinins by absorption and elution and to show that while the purified agglutinins acted most strongly on the red cells used for absorption, they also agglutinated other sorts of cells. Landsteiner concluded that:

> "If one assumes that normal serum contains a sufficient number of agglutinins each reacting with a certain proportion of all bloods, a given sort of blood will absorb from a serum all those agglutinins for which it has affinity and there will remain after absorption some that react with freshly added blood of other species. . . . One may conjecture that there exists a much greater variety of globulin molecules than would appear from physiochemical examination, some of which by virtue of accidental affinity to certain substrates are picked out as antibodies."

The preceding statement by Landsteiner had two of the essential ingredients of a selective theory: natural diversity and accidental affinity. He correctly attributed the specificity of natural antibodies to unique combinations of natural globulins. An extension of this concept to include immune antibodies might have seemed likely, because of the similarity of Landsteiner's own results with antisera to synthetic haptens to those of Malkoff with natural agglutinins (9). However, Landsteiner was not ready to make this jump because a number of essential ideas about cells were missing from his conceptual framework. Immune antibodies were thought

to be more specific than natural antibodies, and no understanding existed of the nature or basis of immunological memory. Biology had not yet entered the immunological arena.

Nevertheless, it was Landsteiner & Chase who started the trend back to cellular studies. Although the current dogma held that natural and immune antibodies were different, it held a very unitarian view of immune antibodies. Agglutinins, precipitins, and hemolysins were considered different manifestations of the same antibody. Thus, when two allergists, Straus & Coca (10), claimed that immediate and delayed hypersensitivities were due to different types of antibodies, Landsteiner set out to prove them wrong. This culminated in the classic report with Chase (11) in 1942 announcing that delayed hypersensitivity could be transferred with cells.

The report of Landsteiner & Chase heralded the rebirth of cellular immunology. Thus, Landsteiner, who had effectively buried Ehrlich's synthesis of cellular and humoral immunity, played a large role in the rebirth of cellular studies 30 years later. The same year (1942) Coons et al (12) introduced the technique of immunofluorescence that made it possible to trace antigen and antibody inside cells. Three years later, Owen (13) reported that cattle twins of different sexes were red-cell chimeras, and six years later (1948), Fagraeus (14) showed that antibodies were made in plasma cells.

Antibody Production

In 1949 Burnet & Fenner (15) published the first edition of a small book called *Production of Antibodies*. In it they strongly attacked the current chemical approach to immunology and the isolation of immunology from the mainstream of biology. They looked to adaptive processes in bacteria for a model of antibody formation and introduced the term "protein synthesizing unit" (ribosomes were unknown at that time). They also attempted to explain Owen's red-cell chimeras with a self-marker theory of immunological tolerance. Thus, the little book played a large role in changing the conceptual framework through which antibody formation was viewed. I know it made a large impression on me, because it was published just as I was starting out in immunology.

It was just at this time that I was fortunate to spend two years (1950–1952) with Frank Dixon learning the new radioisotope techniques and studying the effects of whole-body radiation on antibody formation. In a rooftop laboratory at Washington University Medical School in St. Louis, we labeled bovine gamma globulin with ^{131}I, dialyzed it in huge cellophane sacks, and injected it into hundreds of beautiful white rabbits. We became very adept at removing small samples of blood from rabbit ear veins and then following the disappearance of antigen. The effect of whole-body

radiation was easy to demonstrate. By blocking the production of antibody, radiation prevented the immune removal of labeled antigen from the blood. By varying the time of radiation relative to the injection of antigen, we showed that there were radiosensitive and radioresistant phases in antibody formation. Our explanation of these effects proposed that the antigen-induced modification of the gamma globulin generator was radiosensitive but that the actual production of antibody protein was radioresistant (16). This clearly followed the ideas of Burnet & Fenner.

Using the same ^{131}I–labeled BGG, Paul Maurer and I (17) developed a labeled antigen precipitation test, called the P-80 because the endpoint chosen for comparison of different antisera was the precipitation of 80% of the label. Using this test we could easily show that the quantity of antibody detected in serum depended on a number of factors, such as the serum dilution and the amount of active complement present. This was contrary to the tenets of the "quantitative precipitation test" that purported to measure antibody in absolute nitrogen units. It was the first indication to me that the avidity of antibody was quite variable and that there was no clear line of demarcation between antibody and normal globulin. In other words, the specificity of antibody was not absolute.

In 1952 I moved to the University of Chicago and started to purify labeled antibodies by absorption and elution from an insoluble column. The tremendous variability in avidity of antibodies became quite apparent (18). Some of the labeled antibody came off the antigen column easily, with just a wash. Antibody of intermediate avidity could be exchanged with an unlabeled antiserum or removed with an acid wash. There was always some label that never came off the column. The great variability in avidity seemed to indicate that most antibody made an imperfect fit with antigen. How good did the fit have to be to qualify the globulin as an antibody and what distinguished antibody from nonantibody globulin? Didn't this indicate that there must be a tremendous diversity in normal globulin? If so, was it possible that antibodies were just the rare types of normal globulins that just happened to combine with antigen? These were some of the questions that were raised by the finding that antibodies were highly diverse with respect to avidity.

It seems significant to me that Niels Jerne's thesis work (19) was on the change in the avidity of diphtheria antitoxin and that this probably played a role in his development of the natural selection theory (see below).

In Chicago I developed a close collaboration with William (Tolly) and Lucy Taliaferro. Like Dixon's group, they had also made observations on the radiosensitivity of antibody formation (20). Tolly had started out as a zoologist at Johns Hopkins and had become interested in the host response to parasitic infections. He and Lucy, together with numerous students and

collaborators, had made detailed studies of the cellular responses to trypanosomal and malarial infections. They introduced the term "lymphocyte-macrophage system" to indicate the cells most involved in immunity. Tolly was convinced that lymphocytes were stem cells that could differentiate into other cells such as monocytes, macrophages, and plasma cells. The marked radiosensitivity both of lymphocytes and of the immune response was a strong argument for the importance of lymphocytes to the immune response.

The Taliaferros had switched from studying parasites to measuring the hemolytic antibody response in rabbits to the injection of sheep red blood cells. They had developed an accurate, sensitive, and quantitative assay that could be performed on a small sample of blood. This permitted them to make frequent serial bleedings on the same animal. Using this technique Tolly was able to show that after an injection of antigen the concentration of antibody in the serum rose in a straight line if expressed as the logarithm (21) and that the peak titers of a large group of rabbits showed a log normal distribution.

Both the log normal and log rise of antibody titers seemed to fit with Burnet & Fenner's concept of a natural protein-synthesizing unit that could replicate. It is probably significant that Burnet & Fenner had also noted the logarithmic rise in antibody titer. In 1953, the classic paper of Billingham, Brent & Medawar (22) demonstrated that self-recognition could be learned in utero. This showed that immunological memory could be negative as well as positive and was strong evidence for a genetically determined antibody-synthesizing unit.

In 1954 the Harrises (23) and in 1955 Roberts & Dixon (24) showed that the production of antibody could be transferred with cells. Some of those who supported the antigen-template theory of antibody formation suggested that the anamnestic response and the production of antibody by transferred cells were both due to the release of preformed stores. In 1955 Tolly and I determined to test this hypothesis in a critical experiment (25). A rabbit preimmunized with bovine serum albumin (BSA) was given a second injection of BSA and over the next 5 days, 10 injections of ^{35}S-labeled yeast hydrolysate. At the end of this time the spleen cells of the rabbit were synthesizing highly labeled antibody, as indicated by radioactivity counts in the antigen-antibody complex precipitated from its serum. The spleen cells of this animal were removed, washed thoroughly, and injected into an unlabeled recipient. Within two days the serum of the recipient animal contained measurable amounts of antibody. The fact that this antibody was not labeled was clear evidence that it had been newly synthesized and that we had transferred antibody-producing cells, not preformed antibody. The experiment was also done the other way around (by labeling the recipient

instead of the donor) with the same conclusion. Since only living cells could transfer antibody synthesis in this way, the cells themselves must be the antibody synthesizing units.

Natural Selection Theory

The results of the cell transfer experiments were incompatible with Jerne's Natural Selection Theory (26) which was published the same year (1955). Jerne had postulated a large set of natural globulins that had been diversified in some random fashion. The function of antigen was to combine with those globulins with which it made a chance fit and then to transport these selected globulins into an antibody-forming cell. This cell would then make identical copies of the globulin presented to it.

Jerne's theory was beautiful because it explained most of the recently developed concepts about antibodies. These included (*a*) immunological memory that could be both positive (anamnesis) and negative (immunological tolerance); (*b*) a specificity of antibodies that was not absolute but based on unique combinations of antibodies with a wide spectrum of avidities; (*c*) the identity of natural and immune antibodies; and (*d*) the logarithmic rise in antibody titer and the log normal distribution of peak titers.

However, Jerne's theory had several weaknesses. In addition to its inability to explain the cellular basis of immunological memory, his postulation of protein replication was without precedent and at odds with the developing consensus that the ability to replicate was the exclusive property of nucleic acids. Nevertheless, it was a simple matter to substitute randomly diversified cells for Jerne's randomly diversified globulin molecules and thus to develop a cell selection theory of antibody formation. This appears to have been done independently and almost simultaneously in 1956 and 1957 in Chicago (27) and Melbourne (28).

Evidence in favor of the cell or clonal selection theory was quick to arrive. Nossal & Lederberg (29) reported that single antibody-producing cells in culture made only one antibody. Newborn and fetal pigs (30) were found to have immunoglobulin determinants on the lymphocytes in their circulation. It was possible to destroy selectively the response of spleen cells to one antigen without affecting the response to another (31). Colonies of antibody-forming cells could be found in the spleen of animals given whole body gamma radiation and injected with small numbers of spleen cells (32). Perhaps the most impressive evidence of all was that of Raff, Feldman & de Petris (33), who showed that incubation of lymphocytes with antigen could aggregate (cap) all the surface immunoglobulin on antigen-binding cells; this indicated that the only immunoglobulin on the surface of these cells was antibody of a single specificity.

Despite all the evidence, general acceptance of the cell selection theory was slow in coming. As late as January 1967 I gave a talk to the Midwinter Conference of Immunologists in Honolulu on "The Pallbearers of Clonal Selection" (Figure 1). In it I listed five current arguments against the theory and tried to answer them. Clearly cell selection was still on the defensive despite considerable direct evidence in its favor.

Acceptance of Cell Selection Theory

What happened in the 1970s to change the situation? The theory is not only generally accepted today but has become immunological dogma. This did not happen because of additional direct evidence. I believe it happened because cellular immunology itself exploded. Immunologists began to work with cells. First there were T cells and B cells (34), and then there were ten classes and subclasses of B cells and helper cells and suppressor cells and killer cells. The final coup was production of monoclonal antibodies (35). None of these concepts was designed as a test of cell selection. But the framework on which they were based assumed cell selection.

Molecular immunology also advanced rapidly in the 1970s. The structure of immunoglobulin and its genetic basis were completely developed. The finding that there were many different V genes rearranged randomly in different cells provided the molecular basis for diversity. Cell

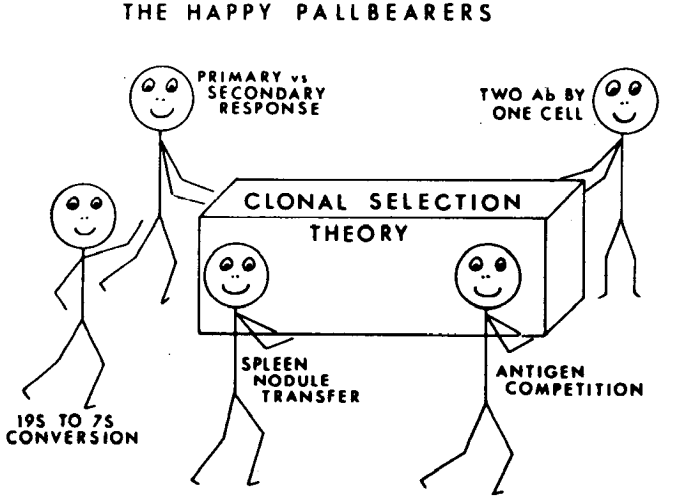

Figure 1 A cartoon used in a talk to the Midwinter Conference of Immunologists in January, 1967, in Honolulu, Hawaii, depicting five current arguments against the clonal selection theory. The conclusion of the talk was that it was too early to bury the theory.

selection ceased to be controversial and became part of the immunological milieu.

CONTEMPORARY CONTROVERSIES

Finally, I would like to mention two concepts that are still quite controversial despite the fact that considerable experimental evidence has been reported in their favor. These are the concepts of an antigen-specific transfer factor and antigen-specific suppressor cells.

The existence of a dialyzable factor that could transfer delayed-type hypersensitivity to a specific antigen was first reported by Lawrence (36) in 1955, two years before the cell selection theories were proposed. At first, transfer factor could be demonstrated only in humans, but more recently it has been repeatedly confirmed in animals (37). One problem with the concept of transfer factor is that a clear picture of what it is and how it works is lacking. Immunologists are unable to connect it with what is known about immunoglobulins and T-cell receptors. The latter is particularly important if transfer factor is responsible for delayed hypersensitivity, an established T-cell function. In this regard, transfer factor lacks the MHC restriction characteristic of T-cells. It is also clearly too small to be the part of either an immunoglobulin molecule or a T-cell that recognizes antigen. Thus, we must assume that transfer factor represents a third type of antigen recognition system. If so, what is its structure and how is its diversity accomplished? Does it have a common and a variable region? These questions will have to be answered before there is general acceptance of the concept.

The problem with suppressor cells is somewhat similar to that with transfer factor, but worse. Despite a plethora of experimental evidence, widespread skepticism remains about the functional significance of suppressor cells. There is not just one antigen-specific suppressor factor, but at least five different factors (38, 39). Suppressor factors appear to come from T-cells and require T-cells to function, but unlike T-cells, they appear to recognize antigen directly (38) i.e. without MHC restriction. In this respect they resemble transfer factor, but unlike transfer factor, suppressor factors are not dialyzable. Thus, suppressor cells require the postulation of a fourth antigen-recognition system.

In summary, transfer factor and suppressor T-cells need to be brought into the mainstream of molecular biology and molecular immunology. I am convinced that they are real, but until their recognition systems and the mechanisms of their action are clearly elucidated, I will be skeptical of their physiological significance.

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Annual Review of Immunology
Volme 4, 1986

CONTENTS

Ann. Rev. Immunol. 1986. 4 : 13–36

FACTORS CONTROLLING THE B-CELL CYCLE

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INTRODUCTION

The basis of a humoral response of the immune system to antigen is the proliferation of antigen-specific B lymphocytes and their maturation to antibody(*immunoglobulin, Ig*)-secreting cells (1). Antigen selects from the repertoire of resting B cells, each expressing one of the diverse sets of variable regions of heavy and light chains of Ig on their surface (2–5). Upon stimulation, in vivo or in vitro, B cells will divide every 20 hr at 37°C, in vitro for 5–15 divisions (6). A single B-cell clone retains remarkable synchrony for several divisions (6, 7). When B cells mature they not only increase their rate of Ig synthesis and begin actively to secrete Ig, they also switch the class of heavy chains that carry the variable regions involved in antigen recognition. A single dividing clone of B cells may switch at any division (8). Switching occurs in individual cells of the clone. Furthermore, a high rate of somatic mutations occurs in Ig genes during the process of clonal B-cell expansion that may alter the specificity of antigen recognition (9–12). Within an expanding clone of B cells, some cells may return to rest and remain in the immune system as long-lived memory cells (13). In vitro such memory cells have not yet been generated unambiguously. In vitro studies with polyclonally activated murine B cells have, however, given some insight into the kinetics of a B-cell response and the qualitative and quantitative changes in the expression of Ig genes that occur during this process (Figure 1) (14).

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B cells can be activated to divide without maturing to Ig secretion (15–17) and can mature without dividing (18–21). In most stimulations, however, successive B-cell cycles are accompanied by gradual increases in the number maturing to Ig-secreting cells (22), a process that appears to be frozen at different stages in different B-cell tumors (23). This complexity and flexibility in responses of B cells to stimulation, whether virgin or memory B cells, make the interpretations of many measurements of B-cell activation ambiguous in their physiological meaning.

For the initiation of most B-cell responses, binding of antigen to Ig on the surface is a necessary but not a sufficient first step. Exceptions to this rule are the polyclonal responses of B cells to lipopolysaccharides (LPS) (24) and to lipid A (25, 26), as well as to lipoprotein (27), that circumvent the binding steps to Ig (28). These responses to B-cell mitogens were early signs that pointed toward our now generally accepted view that antigen-nonspecific growth and maturation factors, binding and signalling via receptors other than surface Ig, are needed to stimulate B cells to proliferate and mature. The other sign that suggested multiple controls of the B-cell cycle came from discoveries indicating that cooperation among helper T lymphocytes, accessory cells (A cells, macrophages), and B cells is needed for B cells

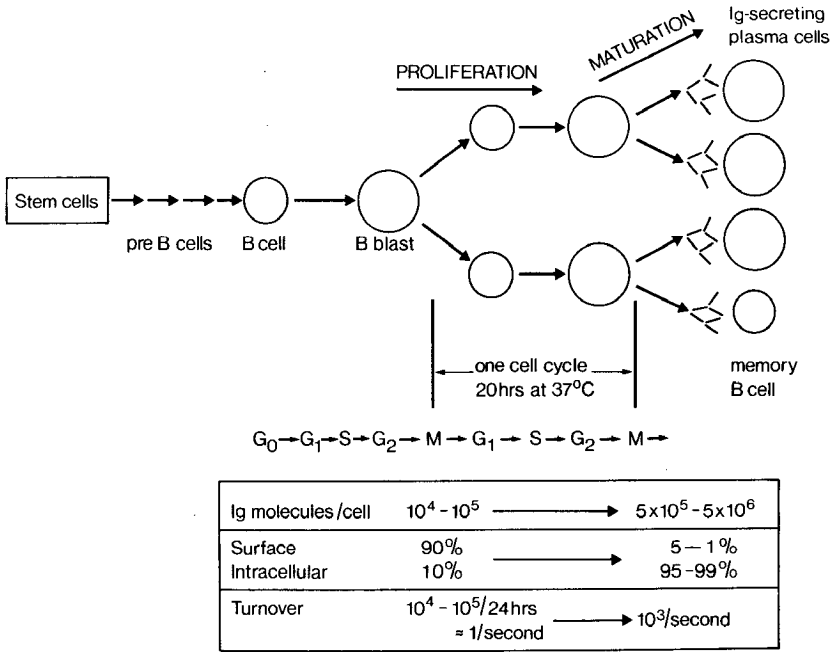

Figure 1 Changes of Ig expression in B cells after stimulation to proliferation and maturation to Ig-secreting cells.

to respond to most antigens (29–32). Furthermore, it became evident that these interactions were governed by the membrane proteins encoded in the major histocompatibility complex (MHC), in particular by those of class-II molecules (Ia-antigens) (33, 34). Thus, only histocompatible cells, i.e. those expressing the same MHC antigens, could cooperate. T cells of a histoincompatible MHC haplotype could, however, adapt during their differentiation from stem cells in the thymus so they could cooperate with A cells and B cells expressing the MHC haplotype that the T cells had encountered in the thymus. Consequently stem cells of (a) haplotype, differentiating in the environment of the (b) thymus, may now cooperate with (b) A and (b) B cells (34, 35). On the other hand, B cells appear to be unable to adapt to a foreign MHC haplotype during their differentiation from stem cells (36), although reports to the contrary exist (37). Thus, interactions between T cells, A cells, and B cells have directions that are imposed by T-cell receptors that interact with antigen in the context of MHC (Figure 2). This concept has gained strong in vitro experimental support by the establishment of antigen-specific, MHC-restricted long-term tissue-culture lines of cloned helper T cells, which have followed these rules for T cell–A cell–B cell interactions (38, 39).

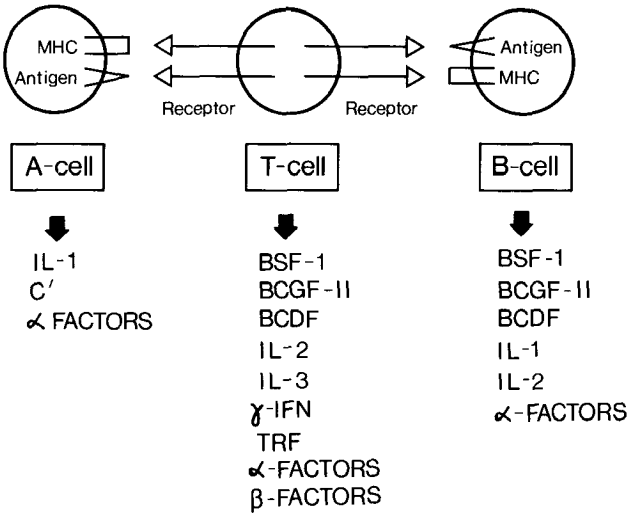

Figure 2 Antigen-specific, MHC-restricted T cell–B cell and T cell–A cell interactions, and lymphokines that have been reported to be produced by the different cells. It should be emphasized that this scheme of lymphokine production is oversimplified as it is derived from measurements with established or transformed cell lines, hybridomas, and tumors, while only scarce information is available for normal resting or activated cells. Our bias is that the spectra of lymphokine production are much more restricted in *normal* A, T, and B cells than those shown here.

The interaction of T cells, A cells, and B cells has at least three phases (40). The first is uptake and presentation of antigen by A cells followed by cell-cell interaction with T cells. This leads to lymphokine production by A and T cells (Figure 2). The second is the excitation of antigen-specific B lymphocytes from their resting state, which makes them susceptible to lymphokine action that induces proliferation. The third is the cell-cycle control of activated lymphocytes by lymphokines, antigen, and cell-cell interactions. Classically, substances that elicit B-cell responses are divided into thymus-independent (41, 42) and thymus-dependent (29–31) antigens on the basis of whether they require cooperating T cells in order to generate an antibody response. Since the final outcome of antigenic stimulation is the same, namely, proliferation and maturation, it implies that thymus-independent antigens can circumvent or substitute for the effects delivered by T cells. We discuss below these multiple effects of the T cells.

THE INVOLVEMENT OF A CELLS IN B-CELL RESPONSES

One of the early events after antigen administration is its uptake by cells of the reticulo-endothelial system. These cells endocytose, digest, and present on the surface the antigen and fragments thereof to the helper T cells that recognize the antigen or its fragments in the context of MHC class-II antigens also expressed on the surface of these so-called antigen-processing, antigen-presenting, accessory (A) cells (43). A variety of cells can perform as A cells for T cells. They all express MHC class-II antigens and have the ability to endocytose, digest, and exocytose the antigen. While macrophages do so irrespective of the antigen, B cells will use their antigen-specific receptors to present "their" antigen specifically. As a consequence, antigen-specific B cells need much (up to 10^4 times) lower concentrations of antigen for presentation (44–46).

One result of the interaction between A cells (or B cells) and T cells is the production of lymphokines that act on T cells and B cells to stimulate proliferation and maturation (Figure 2). The tissue-culture media conditioned by these cell interactions can serve to stimulate antigen-specific B-cell responses in vitro. Since they do so also with T cell–depleted B-cell populations, the factors in these conditioned media have been called T cell–replacing factors (TRF) (47, 48, 112). Considerable confusion exists as to which lymphokines are active on B cells and which cells (A or T cells, or B cells) make which lymphokines. In addition, contaminations of cell lines by mycoplasma may contribute soluble products that may be among the many factors controlling in vitro B-cell growth and maturation (49–51).

It should be stressed that helper-T cells have one direct and two indirect

actions on B cells. In the direct interaction they are expected to excite cells to susceptibility to growth factors by interactions that involve the recognition of antigen and MHC by the T-cell receptor. It remains unclear whether T cell–independent antigens use their regularly repeated determinants to crosslink surface Ig as the T cell–replacing action at this point of the B-cell cycle (52, 53). In the indirect actions two types of factors produced by T cells and by macrophages stimulate B cells through the cell cycle. For the two forms of T-independent antigens, A cells are indispensable for B-cell growth (54). However, differences exist for T-independent antigens in their requirement for lymphokines produced by helper T cells: type-II antigens appear to need them (55), while type-I are independent (52).

THE NATURE OF A CELL–DERIVED B-CELL PROLIFERATION FACTORS

The influence of A cells on B-cell responses can be replaced by that of factors secreted by activated A cells (56, 57). We have called these activities α *factors* (40). Three types of assays have been employed for their detection: (*a*) the proliferation of J558 plasmacytoma cells that require α factor (58), (*b*) the proliferation of A cell–depleted B-cell blasts (59), and (*c*) the activation of A cell–depleted resting B cells into the cell cycle (57). In the two latter assays α factors are required but not sufficient for B-cell proliferation, i.e. they act synergistically together with polyclonal activators such as LPS. Recent studies with A cell–depleted, polyclonally activated, synchronized B-cell blasts have shown that α factors control the entry into S phase at a restriction point some 4–6 hr after mitosis in the G1 phase within the 20-hr-long cell cycle (59). It is at this point that diterpine forskolin may interfere with B-cell growth (60). The action of α factors can be inhibited by the soluble complement component C3d (62). On the other hand C3b or C3d, either aggregated or Sepharose-bound, replaces the effects of α factors in the B-cell cycle (61, 62).

These results recall earlier findings (63, 64) that have implied a role for C3 in the activation of B cells. In line with these findings are observations that find C3d-K, a kallikrein cleavage fragment of iC3b, to be a potent inhibitor of mitogen-induced B-cell proliferation (65). The C3d-specific complement receptor CR2 is expressed lineage-specifically on human B cells as a 140-kd glycoprotein (66–68, 72, 73). Polyclonal and monoclonal antibodies have been generated against CR2. Some of these antibodies have been shown to promote both proliferation and Ig secretion of human B cells (68, 69), while others inhibited such responses (70, 71). Although murine B cells that bind C3 were found enriched in marginal zones (74), the receptors for C3d on murine cells have not yet been characterized in molecular composition and

by specific antibodies (72, 73). One may postulate from these observations that A cells influence B-cell growth by releasing some components of complement—in particular C3, which signals via the C3d-specific CR2 receptor.

The finding that soluble C3d is inhibitory while aggregated C3, C3b, or C3d is stimulatory raises the question why soluble α factors are stimulatory if they are indeed complement components. The hypothesis may be advanced that microaggregation of CR2 receptors could be achieved by C3b interacting with other complement components such as C4b and C2a, thus providing a positive signal for B cells. One may, in fact, expand this speculation by the suggestion that the early pathway of complement activation is enacted on the B-cell surface. It starts with the binding of antigen to surface Ig, which then allows binding of C1q. This, in turn, activates C1r and C1s, then C2, C4, and finally C3. It is conceivable that some of the complement components could even be made by B cells, while others are already known to be produced by A cells such as macrophages (75, 76).

Many transformed cell lines of the T-, B-, and A-cell lineages have been found to produce α-factor-like activities (58), and we suspect that some of the B-cell growth-factor activities reported (77–80) are, in fact, α factors. Furthermore, the B cell growth factors produced by a T-cell hybridoma that stimulate proliferation but no maturation of activated B cells (101) appear to have α-, but no β-factor activity (F. Melchers and W. Lernhardt, in preparation). Interestingly, the Epstein-Barr virus (EBV), which stimulates resting, but not activated, human B cells polyclonally (81, 82), enters the cells via the CR2 receptor (67, 71, 83). The α-factor-like EBV-stimulated entry of human B cells into S phase certainly provides the DNA-synthetic activity of a B cell that is required for the integration of the viral genome. Whether this also is a requisite for the subsequent immortalization of the cells by the virus is not known.

We believe that many of the differences observed in various assay systems for factors influencing B-cell growth and maturation reside in differences in the requirement for exogenously added α factors. Highly purified IL-1 has been shown to have B cell–costimulatory activity (84–86). These experiments should soon also be done with IL-1 made by recombinant DNA technology (87, 88). It may well do so as an α-factor-like activity either directly by binding to the B-cell surface or indirectly by acting on contaminating A cells (57), which in turn produce α-factor activities. It cannot be stressed strongly enough that culture systems for investigating the role of α factors should employ A cell–depleted B-cell populations and must be devoid of serum (89), since serum can be expected to be abundant with such factors. Finally, therefore, it appears that an antigen-presenting

cell must have three properties to elicit a T cell–dependent B-cell response: It must be able to bind (and process) antigen; it must express MHC class-II antigens, and it must produce α-factor activities.

THE NATURE OF T CELL–DERIVED B-CELL PROLIFERATION FACTORS

Both murine and human T-cell lines, clones, tumors, and hybridomas are extensively employed as a source of B-cell growth factors (90–101). Most of these cells must be stimulated by either mitogens (91, 102), phorbol esters (91, 92, 96, 98, 103–106), IL-2 (90), or antigen (97, 106–108) to release such factors, albeit some T-cell lines and tumors secrete them constitutively (98, 109, 110). One well-characterized growth factor is murine BSF-1 (92, 104) to which a monoclonal antibody has been raised (111). BSF-1 can be purified in a series of steps to high purity (93, 104). Even the most purified preparations still elicit both proliferation *and* maturation to Ig-secreting cells of murine B cells in the presence of the appropriate costimulators, i.e. Ig-specific antibodies and fetal calf serum (94, 104). This is not unlike the action of LPS on murine B cells; it also stimulates proliferation *and* maturation. However, this apparently contrasts with findings that proliferation- and maturation-inducing activities are conveyed to the B cells by separate molecular entities (112–114). BSF-1 purified by affinity chromatography over a column with monoclonal antibody is found as two molecular species with weights of 14 kd and 18–20 kd (111). BSF-1 is different from IL-1, IL-2, and IL-3 (93, 115). The action of BSF-1 on B cells can be mimicked by a monoclonal antibody, NIMR-3 (116). Other candidates for such activity may be the antibodies specific for the B cell–differentiation antigen Lyb2 (117) that have been shown to stimulate activated B-cell blasts (118, 119). A monoclonal antibody has recently been found that identifies a potential receptor for BCGF-1 on human B cells (120). It did not induce proliferation of resting B cells but inhibited the BCGF-1–induced proliferation of activated B cells. Our own recent experiments with activated, synchronized murine B-cell blasts have indicated that T cell–derived B-cell replication factors, which we have called β-factors and which may, in part, be BSF-1, act late in the cell cycle in the G2 phase at a restriction point 2–4 hours before mitosis (59).

A B-cell growth factor (B151-TRF) has been purified extensively and shown to retain the capacity to induce proliferation *and* maturation of B cells (109, 169). This activity appears to be different from BSF-1 and, therefore, was suspected to be BCGF-II (121–124). However, B151-TRF differs from BCGF-II by molecular weight and by the capacity to be absorbed by BCL_1 cells (169).

B-cell responses have been divided into thymus-independent and thymus-dependent on the basis of the requirement of the antigens for cooperating T cells. Since the final outcome of these stimulations is the same, namely, proliferation and maturation, it implies that thymus-independent antigens can circumvent or substitute for the effects delivered by T cells. Polyclonal activators such as LPS, and all haptens coupled to them as T-independent antigens of type I, apparently do not need the costimulatory action of BSF-1, β-factors, to stimulate B cells to proliferate. Whether this is also true for T-independent antigens of type II is at least highly questionable since high concentrations of these antigens, in contrast to type-I antigens, are *not* polyclonal activators of B cells (125, 126). Also, B-cell responses to type-II antigens have been found to require factors released by the interaction of cloned helper T cells and A cells (52, 53, 127). In the latter experiments, however, as in many others, no distinction can be made between the factors produced by A cells and those from T cells.

IL-2 IN B-CELL RESPONSES

Both human and murine IL-2 can be obtained in purified form, either by using the isolated genes (128, 129) to produce the protein by recombinant DNA technology or by affinity chromatography using monoclonal anti-IL-2 antibodies (130). When such reagents were used together with monoclonal antibodies that identified the receptor for IL-2 (131–134) [in humans called T-activated cell (TAC)], it soon became evident from a flurry of publications that IL-2 binds to human and murine B cells (135–137), that IL-2 receptor-specific antibodies (139, 140) bind to B cells (138–140), that binding is competitively inhibited by the receptor-specific antibodies, and that B cells have high- and low-avidity binding sites for IL-2 (135, 141, 142), just as T cells do. IL-2 receptors of T and B cells appear to share structural identity (139). There is less agreement as to the functional consequences of this binding (141, 143–146). The antibodies to the IL-2 receptor generally have not induced proliferation or maturation of B cells. The effects of IL-2 on B cells are inhibited by monoclonal antibodies to the IL-2 receptor (139, 140). However, competition of actual binding of these ligands has not yet been shown. IL-2, on the other hand, reportedly induces human B-cell preparations, claimed to be of high purity, either to proliferation (136, 147), to proliferation and maturation (140), or to maturation only (143, 149, 150). For murine B-cell preparations, less agreement is noticeable: While some reports agree with the conclusions reached through studies with human B-cell preparations (137, 144, 145), others show that the proliferation capacity of such B-cell preparations may entirely be due to contaminating T cells (151, 152). That IL-2 is not the only growth factor for B cells is indicated by

the finding that BSF-1 is not IL-2 (93, 153) and that IL-2 can be absorbed out of EL-4 supernatants without loss of B cell–stimulatory activity (143).

T cells and B cells are generally regarded as two separate, however related, lineages of cellular differentiation. The finding that IL-2 has receptors on B cells and acts in functional ways with these cells appears as a departure from the notion that cell lineages can be distinguished by cell lineage–specific growth factors and corresponding specific receptors. We must leave it to a more specified review, in which the purity of B-cell preparations, the purity of IL-2 and IL-2-receptor-specific antibodies, the specificity of binding of these ligands, the crossreactions between species (e.g. human IL-2 on murine B cells), and the quantitation of the functional responses and their in vitro conditions are critically assessed, to accept the notion that, as one example, a murine B cell will proliferate (and mature) in response to either BSF-1 or to IL-2.

MATURATION FACTORS FOR B CELLS

γ-Interferon, made by recombinant DNA technology, has been implicated as a factor that induces the maturation of B cells to Ig-secreting cells (147, 148). Recently, however, reports increase that researchers cannot see this activity with B cells (93, 115, 124). It has been claimed that factors inducing proliferation are different from those inducing maturation (113, 114), but BSF-1, as purified as it can be obtained, still induces both proliferation and maturation of B cells (94, 104, 105, 115, 169). Maturation factors inducing IgM secretion have been found to be different from those inducing IgG secretion (154–156), but the same highly purified preparations of BSF-1 and of B151-TRF induce IgM as well as IgG secretion (104, 105, 157, 158; K. Takatsu, F. Melchers, in preparation). Furthermore, IL-2 can induce maturation (150), although the maturation factors for IgM and IgG reported in previous publications are not IL-2. All these apparent disagreements and paradoxes, to our mind, demonstrate the complexity of the controls that a B cell uses to mature to a secreting cell. It appears too simple to hope that a single molecule and its corresponding receptor may regulate these different manifestations of the process of maturation.

CELLULAR SOURCES OF LYMPHOKINES

Clones of helper T cells, T-cell tumors, and T-cell hybridomas have helped to clarify the fact that one cell can make a plethora of lymphokines. Lymphokines produced by T cells, T-cell tumors, or T-cell hybridomas include BSF-1 (90, 92–96, 159), BCGF-II (92, 95, 97–101, 121, 123, 124), γ-interferon (160–163), IL-2 (164), IL-3 (165, 166), TRF (95, 98, 102, 105, 109,

115, 150, 167–169), BCDF (104, 168), β factors (59), and α factors (58). Those made by B cells or B-cell tumors include BSF-1 (77, 79, 80), BCGF-II (78), BCDF (169), α factors (58), IL-1 (170), and even IL-2 (171). Those made by A cells include IL-1 (85, 86), complement components (76), and α factors (57). Thus A cells appear to produce a spectrum of lymphokines that is different from those of T and B cells or their tumors, while T cells and B cells or their tumors produce a widely overlapping spectrum of lymphokines. α Factors can be produced by all lineages (58). It remains unclear whether a difference can be seen in the spectrum of lymphokines produced by *normal* lymphocytes when compared with the one produced by established cell lines and their transformed and malignant counterparts.

THE RESTING B CELL

A resting B cell in the G0 phase of the cell cycle is refractory to the action of B cell proliferation–inducing factors (39). While most investigations agree with this notion (40, 92), some do not (106, 107, 172). A resting B cell can, however, be stimulated to maturation without proliferation (21). There are several reports that confirm these findings, some with more purified preparations of factors (96, 173). Again, purified BSF-1 appears to induce this maturation without proliferation (96).

B cells have to be excited from their resting state to become susceptible to the action of the proliferation-inducing factors. It remains to be seen whether the receptors for these factors are only expressed on the surface of B cells after excitation, or whether the receptors are always there but only after excitation become functionally linked to reactions that induce the mitotic cell cycle. At least, a number of surface markers appear on activated cells that cannot be detected on resting cells (174–176). Among those are B1 molecules on human B cells. Monoclonal antibodies against B1 inhibit B-cell proliferation (175). There are multiple ways to excite a resting B cell. T cell–dependent, alloreactive T cells recognize the foreign MHC antigens on B cells (177, 178), while antigen-specific, MHC–restricted T cells recognize the antigen in context with self-MHC on the B cell (39, 45, 178) (Figure 2). There is no general agreement on how important the latter recognition is in the total repertoire of T-B interaction in a mouse or in other species. Lyb5$^+$ as well as Lyb5$^-$ B cells can be activated by MHC-restricted, as well as MHC-unrestricted, pathways with the help of a single helper T–cell clone, and this may be due to a difference in the cell-cycle state of these B cells, i.e. either resting or activated (179). The combined findings of many laboratories experimenting with many different T cells indicate that MHC class-II antigens play an important role in the excitation reaction. Furthermore,

antibodies have been raised to class-II MHC antigens that either stimulate B cells (180) or inhibit LPS-induced stimulation of B cells (181). It remains, therefore, all the more remarkable that Schimpl & Wecker's T cell–replacing factors (TRF) (112) can activate sheep erythrocyte–specific B cells in the presence of antigen, but in the apparent absence of MHC-recognizing structures. While the search for such antigen-specific, MHC-restricted structures has been frustrating for many (40), the hope is that such structures are, in fact, the T-cell receptors themselves and should be available soon in purified forms, produced by recombinant DNA technology.

B cells can also be excited from the resting state in MHC-unrestricted ways by either Ig-specific antibodies (182–185), usually in crosslinked form, or by polyclonal activators such as LPS, or by phorbol esters (136, 185, 186). Monoclonal antibodies directed towards any of the four constant region domains of the μ heavy chain will serve as acceptor sites for the polyclonal excitation by monoclonal antibodies (187, 188). Recently, mutant EL-4 thymoma lines have been generated that activate B cells by direct cell-cell contact polyclonally (189). It remains to be seen whether these interactions are dependent on MHC recognition while not being selective for a given MHC allele.

We have mentioned above the possible role of T cell–independent antigens as molecules replacing the action of B-cell growth factors in the cell cycle, i.e. the action of BSF-1 or β factors. A second function of T cell–replacing activity of T cell–independent antigens becomes apparent in the excitation of B cells from their resting state. A long-held belief is that ordered crosslinking of surface Ig on B cells by regularly repeated determinants of T cell–independent antigens is obligatory for stimulation. It may be at this point of excitation into G1 that this crosslinking plays a crucial role. Binding of antigens with single determinants may only induce crosslinking of surface Ig when a T cell recognizes a carrier determinant in the context of MHC class-II antigens. It thereby crosslinks the surface Ig molecules by multiple interactions with a multitude of T-cell receptors; T cell–independent antigens of types I and II may achieve this crosslinking without the help of multiple T-cell receptors in the membrane of a T cell. It may turn out to be the common denominator in the action of T cell–independent antigens and of T cells with B cells and may one day clarify one of the controls that regulates B-cell growth.

The final cell response of maturation only, or of proliferation and maturation, can be quantitated (6, 190). The proliferative response is quantitated by limiting dilution analysis (6). Even the most potent polyclonal activators have never been found to activate *all* B cells—at best,

30–40% of them (6). The same holds for maturation of B cells without proliferation (21). When tissue culture systems are employed that either have no filler cells (191, 192) or replace the usual thymus filler cells by 3T3-fibroblasts (193), the response is at best with 3–5% of all B cells and must be aided by high concentrations of fetal calf serum and polyanions. Nevertheless, these culture systems allow quantitations of B-cell responses that should be adequate references for comparison of data from different laboratories. This, unfortunately, is often not done and thus makes differences and paradoxes in experimental findings difficult to resolve.

THE TRANSITION FROM G0 TO G1 IN B CELLS

A wealth of early changes in biochemical parameters of B cells has been observed after they have been stimulated with polyclonal activators and with Ig-specific antibodies. Changes include increases in the size of B cells (94, 96, 194, 195), increased Ca^{++} uptake (196), depolarization of surface membranes (196), changed phosphoinositol metabolism (194, 197), increased Ia (175, 198) and other surface marker (174–176) expression, and increased RNA constant (195). In some experimental conditions, factors produced by cloned helper T cells induce resting B cells to increased Ia expression and RNA synthesis as well as to entry into S phase (172). Many of these changes can be monitored at the single cell level by fluorescence-activated cell analysis. It is remarkable that several of these early changes can be detected in practically *all* B cells of a given preparation. This is in marked contrast to the subsequent, much lower capacity of the same cell population to enter the mitotic cycle (6). Furthermore, many of the changes occur very rapidly and apparently synchronously, again in marked contrast to the finding that entry into the mitotic cycle is asynchronous and occurs at a constant rate after activation into G1 for the first 24 hours (7). Consequently early states of activation into G1 can be distinguished from late states by RNA content and surface marker expressions of B cells (195, 198). For the present time we are unable to link a given early change of B cells to reactions that finally lead to either proliferation alone, maturation alone, or to both. Purified BSF-1 activates resting B cells to an enlarged, G1-like state *before* the action of Ig-specific antibodies. The apparently paradoxical effect of this could be interpreted as a reaction that would finally lead not to proliferation but to maturation without proliferation (94). It may, in fact, not lead to any further cell-cycle progression at all. The dissection of the B-cell cycle into three restriction points (59), mentioned below, may help to order at least some of these early reactions and their functional consequences for B-cell proliferation and maturation.

CELL-CYCLE CONTROL OF ACTIVATED B CELLS

Studies with LPS as a polyclonal stimulator of multiple cell cycles have shown that individual B-cell clones at 37°C divide synchronously every 18–20 hr for at least 5, and in most cases no more than 7–10, divisions (6, 7). Recently, it became possible to synchronize asynchronously activated mass cultures of A cell–depleted B cells by size selection using velocity sedimentation (59). To enter several subsequent cycles, B-cell blasts of a given size were shown to require macrophage-derived α factors, helper T cell–derived β factors, and Sepharose-bound, Ig H chain–specific monoclonal antibodies. LPS was shown to replace the action of both Ig-specific antibodies and β factors (59), while α factors could be replaced by Sepharose-bound or glutaraldehyde-crosslinked C3b and C3d (62). Nonoverlapping, synergistic effects of LPS or a specific T_h signal on the one hand, and Ig-specific antibodies on the other, have also been observed in the induction of growth-factor responsiveness of B cells (103). When α factors (or C3) were omitted, B cells were arrested within the G_1 phase of the cell cycle, before entry into S phase. It is worth noting that this effect of α factors is similar to that of insulin-like growth factors, i.e. of progression factors,

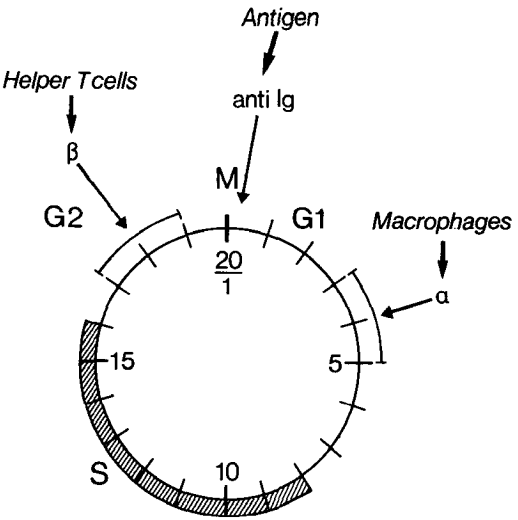

Figure 3 The 20-hr cell cycle of activated murine B lymphocytes, divided into 8 hr of G1-phase, followed by 8 hr of S-phase, then by 4 hr of G2-phase and finally by mitosis (M). In this cycle are placed the three restriction points controlled by Ig-specific antibodies (anti-Ig); α factors (α); and β factors (β). α Indicates the point of influence of macrophages, β that of helper T lymphocytes, and anti-Ig that of antigen in immune responses of B cells (55).

that control the entry into S phase of other eukaryotic cell lineages. There is very weak homology in the nucleotide sequence for C3d within the α chain of C3 (199) to murine epidermal and nerve growth factors; this suggests a possible phylogenetic relationship of C3d with insulin-like growth factors (62). When β factors were omitted, the B cells became arrested in the G2 phase of the cell cycle, 2–4 hr before mitosis. This rather unusual restriction point in the cell cycle, controlled by T cells, is at a stage when B cells have two sister chromatids. If sister chromatid exchange is required for class switching (11), then a delay in the provision of T-cell help may facilitate class switching in activated B cells and, thus, provide an explanation for the influence of T cells on Ig class switches (200, 201). When Ig-specific antibodies were omitted, cells became arrested directly after mitosis, early in G1. It therefore appears that a reaction similar to the original excitation reaction may be needed for the beginning of every new B-cell cycle. It remains to be investigated why earlier studies with different factor preparations and with asynchronous A cell–containing, B-cell blasts (6, 7) allowed B-cell blasts to divide several times in the absence of Ig-specific antibodies (101, 202–204). Three restriction points in the B-cell cycle further support the idea of multiple controls in B-cell growth, effected by antigen, macrophages, and helper T cells. These controls may become deregulated in different ways and different combinations to lead to autoimmunity, immunodeficiency, and malignancy (205–207).

COMMENTS AND ACKNOWLEDGMENTS

Several excellent reviews (40, 154, 208–211) have appeared lately—two of them in this series (154, 209)—that have covered large parts of the history and the recent (to that point) literature of the various aspects of B-cell activation and B-cell growth factors. Our attempt here has been to update the literature collection on B cell–growth control. We have found more than 150 pertinent papers for the years 1984 and (up to June) 1985. We apologize here to all our colleagues whose publications we have missed. We have taken this review also as an opportunity to summarize what we hope are accepted facts of the field, to point out controversies and paradoxes, and to state our biases. We have found it impossible to compare critically the experimental data of the papers and that of the literature already existing in the field, and thus to judge the validity of all the conclusions we quote.

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CONTENTS

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AN OVERVIEW OF T-SUPPRESSOR CELL CIRCUITS[1]

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INTRODUCTION

T-suppressor circuits show many similarities to conventional positive immune responses. They are no more complicated than effector systems

[1] Abbreviations used in this chapter: ABA, azobenzenearsonate; Ag, antigen; Con A, concanavalin A; DNP, dinitrophenyl; FcR, receptor for Fc of immunoglobulin; GT, GAT, copolymers of glutamic acid and tyrosine and glutamic acid, tyrosine, and alanine; IL-2, interleukin 2; KLH, keyhole limpet hemocyanin; MHC, major histocompatibility complex; LDH, lactate dehydrogenase; NP, 4-hydroxy-3-nitrophenylacetyl; PSA, picrylsulfonic acid, 2,4,6-trinitrobenzenesulfonic acid; T-acc, T-acceptor cell; Th, T-helper cell; ThF, antigen-specific T-helper factor; TMA, phenyltrimethylammonium; TNP, trinitrophenyl or picryl; Ts, T-suppressor cell; TsF, T-suppressor factor, antigen-specific unless otherwise stated; Ts-aff, TsF-aff, T-suppressor cells and factors afferent, which only act when given early in the immune response. They differ from Ts-ind by not acting through the Ts-eff/T-acc circuit; Ts-eff, TsF-eff, antigen-directed Ts and TsF effector which act at the effector stage of the immune response; Ts-ind, TsF-ind, Ts and TsF inducer that induce idiotype-directed Ts-trans and antigen-directed Ts-eff; Ts-trans, TsF-trans, Ts and TsF are anti-idiotypic and act on the Ts-eff.

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such as antibody, which augments and depresses immune responses and has modes of action through the mast cell and the complement cascade. Moreover, the induction of T-suppressor cells by antigen in the context of I-J is analogous to the induction of conventional responses by antigen in the context of I-A, while the sequence of T-suppressor-inducer and T-suppressor-effector cells is analogous to conventional T-T or T-B collaboration.

This review is mainly concerned with T-suppressor cells (Ts) that affect contact sensitivity in the picryl (trinitrophenyl, TNP) and oxazolone (4-ethoxymethylene-2-phenyloxazolone) systems and compares these with a synoptic view of other systems, many of which have been reviewed in this series (1, 2, 3, 4, 5). It starts with a discussion of nomenclature and continues with a comparison of antigen-specific T-helper factors (ThF) and antigen-specific T-suppressor-inducer factors (TsF-ind or TsF_1), which may be regarded as antigen-specific "helper factors for the induction of suppression." This discussion relates our knowledge of the structure and recognition sites on antigen-specific ThF to its biological properties. The next part deals with the expression stage of the T-suppressor cell circuit and elaborates the role of the T-acceptor cell (T-acc), which produces non-specific T-suppressor factor(s) (TsF) that inhibit the passive transfer of contact sensitivity and the production of IL-2. A discussion of the possible effects of IL-2 on the induction and maintenance of tolerance links this modulation of IL-2 production to the general problem of immunological unresponsiveness. The review continues with a description of other T-suppressor-afferent cells (Ts-aff) that limit the response to contact sensitizing agents but are different from Ts-ind (Ts_1) and do not act through the Ts effector (Ts-eff, Ts_3). Their existence emphasizes the need to explore the response to different antigens and different modes of immunization with an open mind so as to discover how many different systems exist. The review ends with a general consideration of the biological role of T-T collaboration and anti-idiotype loops in helper and suppressor circuits.

Mode of Action of T-Suppressor Factors

A rational nomenclature for suppressor cells requires some knowledge of how suppressor cells act. The main modes of action of the cells and factors in suppressor cell circuits are:

1. Interference with the induction of immunity or unresponsiveness at the level of the antigen-presenting cell—a phenomenon sometimes called immunological agnosis (6);

2a. Provision of antigen-specific help for the induction of suppressor cells which, together with antigen, gives rise to an antigen-directed Ts-eff by

a process of immunization, as in the lysozyme (7, 8) and GT (9) systems; cf antigen-specific help for T-cell cytotoxicity, delayed and contact sensitivity (10; see 11);

2b. Provision of help and idiotype (antigen) for the production of idiotype-specific Ts-transducer (Ts-trans), as in the NP and ABA (Ts_2) systems (2, 12);

3. Provision of antigen-nonspecific proliferation or differentiation factors (13, 14);

4. Activation of Ts-eff to secrete Tsf-eff when stimulated by anti-idiotypic TsF-trans. In this context TsF-trans may be regarded as an internal image of antigen and hence as able to stimulate the Ts-eff (15);

5. Direct action of TsF on target cell. This is proposed in the LDH system (16);

6. Arming of nonspecific acceptor cells, such as the T-acceptor cell or macrophages. These cells, when subsequently exposed to antigen and I-J, release nonspecific TsF (17) and macrophage suppressor factor (18). Both suppressor factors block the transfer of contact sensitivity (19), and the nonspecific TsF also blocks the production of IL-2 (20, 21).

Another way of looking at the T-suppressor-cell circuits is to consider the nature of the interactions between the different cells. There are four main interactions: *immunization*, *immunization with T-cell help*, *triggering* of specific cells to release antigen-specific TsF and perhaps nonspecific factors, and *arming* of cells with specific TsF and the subsequent *triggering* of the release of nonspecific factors by antigen and I-J (22). The initial *immunization* to produce Ts-ind (Ts_1) can be effected by haptenized I-J^+ macrophages (23) and in some systems by haptenized I-J^+ T cells (24).

In some systems the complete Ts-eff/T-acc circuit occurs without any anti-idiotypic loop. This is seen in the lysozyme, GT (7, 8, 9), and picryl system (25) [when picrylated cells are used to induce Ts]. Antigen, together with TsF-ind (TsF_1), induces antigen-directed Ts-eff cells by a process of *immunization together with T-cell help*. This Ts-eff is usually called Ts_2. In other systems, the complete Ts-eff/T-acc circuit with an anti-idiotypic loop occurs. Thus, in the NP and ABA systems (2, 12) the TsF-ind (TsF_1) provides both help and the antigen (idiotype) for the induction of the anti-idiotypic Ts-trans (Ts_2). In this case the anti-idiotypic TsF-trans *triggers* the release of TsF-eff from the Ts-eff that arises independently (2). Although this Ts-eff is equivalent to the Ts-eff in the lysozyme system, it is usually called Ts_3. In the picryl, oxazolone (18, 26), NP (27, 28), and lysozyme (7) systems, the Ts-eff has a mode of action by *arming* a T-acceptor cell or macrophage. Finally, antigen together with I-J *triggers* the release of nonspecific TsF from the armed T-acc. In some systems the circuit is

apparently incomplete and there is no evidence at present for Ts-ind that augment the production of Ts-eff. Thus, although cells making TsF-ind are produced by the injection of picrylated cells intravenously (25), there are no data on whether similar cells are involved when Ts-eff are induced by the injection of the water soluble, chemically reactive haptens, picrylsulfonic acid (trinitrobenzenesulfonic acid, PSA) and "oxazolone-thioglycolic" acid (29). In fact, the complete circuit is apparently absent, and only the Ts-eff and the T-acc have been described. Whether these Ts-eff are produced without T-T collaboration or whether Ts-ind remain to be discovered is unknown. These systems are summarized in Table 1.

Nomenclature

The nomenclature of T-suppressor cells and factors is somewhat confusing because the term Ts_2 has two different meanings and has been used to refer both to the antigen-directed Ts-eff and to the idiotype-directed Ts-trans that act on the Ts-eff. In fact, Benacerraf's terms (30), Ts_1, Ts_2, and Ts_3, correspond to Gershon's terms Ts-ind, Ts-trans, and Ts-eff (1). Ts_1 and Ts_3 have also been called Ts afferent and Ts efferent (4, 5, 31). Both nomenclatures leave open the nature of the interactions between the cells. Because of the confusion over Ts_2 and Ts_3 we wish to propose the use of the terms *Ts-inducer* (if necessary specifying whether for the induction of Ts-trans or Ts-eff) for the cell that behaves like a T-helper cell for the induction of suppressor cells; *Ts-transducer* for the anti-idiotypic cell whose TsF triggers the Ts-effector cell; *Ts-effector* for the cell that makes antigen-specific TsF-eff; and *T-acceptor* cell for the cell armed by TsF-eff that releases nonspecific inhibitors when exposed to antigen. The T-acc is also known as the Ts auxiliary cell or Ts_4 (28, 32). This nomenclature is summarized in Table 2.

T-Suppressor Afferent Cells

In both the lysozyme and NP circuits there is a cell, the Ts-ind, that suppresses only when given early and acts through the Ts-eff/T-acc circuit. Ts-eff and Ts-ind also occur together in the picryl system (25). However, other T-suppressor cells are found in the picryl system that like the Ts-ind (Ts_1), only inhibit when given early but, unlike the Ts-ind, do not act through the Ts-eff/T-acc circuit (5). These cells may be termed Ts-afferent, in contradistinction to the Ts-ind of the previous circuit. Many specific suppressor cells that affect cell proliferation (36) and the induction of antibody and delayed hypersensitivity (35) may belong to this family of cells (see 5). The evidence for the existence of these distinctive cells is discussed in the section on T-suppressor afferent cells.

Table 1 T-suppressor inducer, transducer, effector, and acceptor cells and their interactions

Circuit	Activity	Target cell	Result
Complete Ts-eff/T-acc circuit with anti-idiotypic loop, e.g. NP system	(*a*) Induction of Ts-ind[a] by ag on I-J$^+$ cell [*Immunization*]	Unprimed Ts-ind	Primed Ts-ind (Ts_1)[a]
	(*b*) Induction of Ts-trans[b] by TsF-ind[a] [*Immunization* with TsF-ind providing idiotype and T help]	Unprimed Ts-trans	Primed Ts-trans (Ts_2)[b]
	(*c*) Induction of Ts-eff[a] by ag on I-J$^+$ cell [*Immunization*]	Unprimed Ts-eff	Primed Ts-eff (Ts_3)[a]
	(*d*) *Triggering* of Ts-eff[a] by TsF-trans[b] to produce TsF-eff[a]	Primed Ts-eff	Release of TsF-eff (TsF_3)[a]
	(*e*) *Arming* of acceptor cell with TsF-eff[a]	T-acceptor cell Macrophage	Armed T-acc (Ts_4) Armed macrophage
	(*f*) *Triggering* of acceptor cell by ag and I-J	Armed T-acc Macrophage	Release of nonspecific TsF or macrophage suppressor factor
Complete Ts-eff/T-acc circuit without anti-idiotypic loop, e.g. lysozyme system	(*a*) Induction of Ts-ind[a] by ag on I-J$^+$ cell [*Immunization*]	Unprimed Ts-ind	Primed Ts-ind (Ts_1)[a]
	(*b1*) Induction of Ts-eff[a] by TsF-ind[a] + ag [*Immunization* with TsF-ind[a] providing T help]	Unprimed Ts-eff	Primed Ts-eff (Ts_3)[a]
	(*e*) *Arming*	Acceptor cell	Armed acceptor cell (Ts_4)
	(*f*) *Triggering*	Armed acceptor cell	Nonspecific inhibitors
Incomplete Ts-eff/T-acc circuit without known Ts-ind, e.g. picryl (picrylsulfonic acid) system	(*c*) Induction of Ts-eff by ag on I-J$^+$ cell [*Immunization*]	Unprimed Ts-eff	Primed Ts-eff (Ts_3)
	(*d1*) *Triggering* of Ts-eff by antigen + I-J to produce TsF-eff	Primed Ts-eff	Release of TsF-eff (TsF_3)[a,c]
	(*e*) *Arming*	Acceptor cell	Armed acceptor cell (Ts_4)
	(*f*) *Triggering*	Armed acceptor cell	Nonspecific inhibitors
Possible direct effects of TsF-eff	(*g*) *Inhibition*	T cell	Inhibition of proliferation, lymphokine production
		B cell	Inhibition of antibody production

[a] Directed against antigen.
[b] Directed against idiotype.
[c] Ts-eff secretes the antigen-binding chain of TsF even without stimulation by additional antigen. However, antigen together with I-J is needed for the secretion of high molecular weight, disulfide bonded TsF.

Table 2 Summary of nomenclature of T-suppressor circuits

Name	Synonym	Directed against	Mode of action	Examples
Ts inducer (Ts-ind)	Ts_1 Ts afferant	Antigen	Provides idiotype for inducing second order Ts-trans directed against idiotype	NP (2), ABA (12), TMA[c] (33)
			Provides I-J that together with antigen induces second order Ts-trans directed against antigen	Lysozyme[b] (7), picryl[d] (25), GT, GAT (34)
Ts transducer (Ts-trans)	Ts_2	Idiotype	Triggers Ts-eff to release antigen-specific TsF effector	NP (2)
Ts effector (Ts-eff)	Ts_3[a] Ts-efferent	Antigen	Arms: macrophage	Picryl (18)
			T-acceptor cells	Picryl, oxazolone (26), NP (27), lysozyme (7)
T acceptor (T-acc)	Ts_4 Ts auxiliary		Releases nonspecific inhibitor when triggered with antigen and I-J	Picryl, oxazolone (26), NP (27), lysozyme (7)
Ts afferent (Ts-aff)			Unknown. Does not have an obligatory mode of action through the Ts-eff/T-acc circuit	Picryl (34, 35), DNP (30)

[a] Called Ts_2 in systems in which the Ts-ind together with antigen generates Ts-eff.
[b] I-J genetic restriction.
[c] Both antigen and Ts-ind required.
[d] MHC genetic restriction. The other Ts-ind are not MHC (I-J) genetically restricted in their action.

THE ANALOGY BETWEEN ANTIGEN-SPECIFIC T-HELPER FACTOR AND T-SUPPRESSOR INDUCER AND EFFECTOR FACTORS

This section compares antigen-specific ThF with the antigen-specific TsF-ind that acts by providing help for the production of Ts-trans and Ts-eff. However, the structure of TsF-eff closely resembles that of ThF and TsF-ind and is also discussed here.

Production of Antigen-Specific T-Helper Factor

Antigen-specific ThF occurs in the culture supernatants of nylon-wool-purified, lymph-node T cells from mice painted with contact sensitizer, e.g. picryl chloride. It increases the contact sensitivity response to a suboptimal dose of picrylated lymphoid cells that have been incubated in it and then injected into the footpad. It is one of several antigen-specific ThF that affect cell-mediated immunity (10; see 11). It probably differs from the mast cell–arming factor that causes ear swelling in mice (37), as this is genetically unrestricted in its action, while ThF is I-A restricted (38).

Minimal Model for T-Helper Factor

It may be useful to summarize the structure of ThF and TsF and to give the qualifications afterwards. ThF consists of two chains, one that binds antigen and another that contains I-A determinants (see Figure 1). After dissociation by reduction, the two chains can be separated. Both are needed for biological activity, and there is an I-A genetic restriction in this interaction (39). For this reason the antigen-binding chain is shown as having a recognition site for I-A. Antigen-specific TsF is similar except that it bears I-J determinants, and the restriction between the chains maps to I-J in the one case studied (40, 41).

T-HELPER FACTOR BINDS TO ANTIGEN The antigen specificity of ThF and affinity chromatography on picryl linked to Sepharose through diamino-hexane show that ThF has an antigen-binding site and that the binding

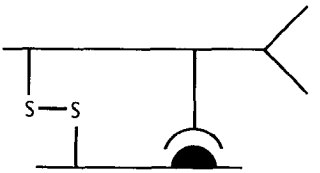

Figure 1 Minimal model for antigen-specific T-helper factor. The upper chain with its antigen-binding site (angle) is disulfide bonded to the lower chain with its I-A determinant (black half-moon). Note the interaction between the antigen-binding chain and the I-A determinant shown by the open half-moon. In general, antigen-specific T-suppressor factor is similar, but I-A is replaced by I-J.

does not require a protein carrier or MHC (11). Similarly, TsF-ind in the NP (2), ABA (see 12), KLH (42), and lysozyme systems (7, 8) and TsF-eff in the picryl, oxazolone (29), and NP (2) systems bind directly to antigen. Moreover, most antigen-directed T-suppressor cells adhere to antigen-coated plates. However, in the pneumococcal polysaccharide system, association of the antigen with I-J determinants is required for adherence (43). Perhaps the affinity of binding of the suppressor–T cell receptor to the hydrophilic polysaccharide is insufficient, and binding to I-J is also required. The role of positive cooperativity in the binding of antigen and MHC, in which the binding of one increases the affinity for the other, may be relevant here (44).

I-A$^+$ T-HELPER FACTOR AND I-J$^+$ T-SUPPRESSOR FACTOR Affinity chromatography on monoclonal antibody shows that picryl and other ThF bear at least some I-A determinant(s) (3, 11, 45, 46). However, the occurrence of I-A determinants may not indicate the presence of a complete chain of conventional I-A. Likewise, nearly all TsF studied have I-J determinants or require a separate I-J$^+$ chain for their activity. There are no unequivocal exceptions. The finding that TsF in the DNP and schistosomal (4, 47) systems bears I-C determinants needs reassessing in the light of current views on class-II antigens (48). In the picryl system, disulfide bonded TsF-eff contains an I-J$^+$ chain (40); the reports of I-J$^-$ factor are probably due to studying free antigen-binding chain instead of the complete disulfide-bonded molecule (49). Finally in the LDH system, monoclonal TsF consists of an antigen-binding chain linked with a chain bearing A_β or E_β determinant(s). However, the anti-A_β monoclonal used crossreacts with I-J, while certain anti-I-J react with E_β determinants (16). The occurrence of I-J determinants on antigen-specific TsF and on certain nonspecific TsF (50, 51) suggests that cell surface receptors for I-J may be important targets for the action of these factors.

ANTIGEN-SPECIFIC T-HELPER AND T-SUPPRESSOR FACTORS HAVE A TWO-CHAIN DISULFIDE-BONDED STRUCTURE Reduction and alkylation under mild conditions splits picryl-specific ThF into two chains: one chain is antigen binding, and it is presumed that the other chain, which is antigen nonbinding, bears the I-A determinant(s) (39) (see Figure 1). T-cell augmenting factor in the KLH system has a similar structure (46). On the suppressor side, TsF-ind has a disulfide-bonded, two-chain structure with one chain binding antigen and the other chain bearing the I-J determinant. This is seen in the KLH (42, 51) and GT (52) systems. Likewise TsF-eff has a two-chain structure in the picryl (40), NP (53), GAT (34), and LDH (54) systems.

The biological activity of disulfide-bonded TsF apparently varies in its

susceptibility to reduction and alkylation. This may be due to the amount of oxygen in the solutions used, as there is a critical intrachain disulfide bond that cannot reform when reduction and alkylation occur under anaerobic conditions (54). In keeping with this, retention of activity after reduction is not due to reassociation of the chains (34).

SINGLE-CHAIN, ANTIGEN-SPECIFIC T-SUPPRESSOR FACTOR Not all factors have a two-chain disulfide structure. In some helper (46) and suppressor systems the two chains occur separately, and both are needed for biological activity (40, 41, 42, 55). There are also clear descriptions of a single-chain, biologically active Ts-ind with both an antigen-binding site and I-J determinants in the GAT (9, 34) and TMA (33) systems. These induce Ts-eff and Ts-trans, respectively. There are no descriptions of similar TsF-trans or Ts-eff.

Complementation Between the Two Chains of Antigen-Specific Factors

BASIC PHENOMENON Both chains of ThF are required for biological activity. However, unlike most antibodies, the antigen-nonbinding chain is not an essential part of the antigen-binding site. Thus, the antigen-binding chain of picryl ThF can be complemented by the antigen-nonbinding chain of oxazolone ThF (39). Similarly, the antigen-binding chain of TsF-eff, which occurs free in mice injected with PSA without painting, can be complemented by I-J from nonimmune cells (41). Complementation also occurs between the antigen-binding chain and an I-J$^+$ antigen-nonbinding chain from several sources in the sheep red blood cell antibody system; this again shows that the I-J chain does not make an essential contribution to the antigen combining site (55).

GENETIC RESTRICTION IN THE INTERCHAIN COMPLEMENTATION, INDUCTION, AND ACTION OF ANTIGEN-SPECIFIC FACTORS An interesting generalization can be made concerning the role of the I-A region of the MHC in relation to ThF. In fact the MHC determinant(s) of ThF, and three genetic restrictions, i.e. in the ability of the separated chains of ThF to show biological activity (interchain complementation), in the induction of ThF by picrylated cells and in the action of ThF, all map to the I-A region. A similar generalization applies to TsF, except that the mapping is to the I-J region. One exception is the Ts-eff in the DNP system, which is class-I restricted but bears class-II determinants (55a). The most likely explanation of this generalization is that the antigen-binding chain of factors and the T-cell receptor have recognition sites for MHC-related determinants: I-A for Th and I-J for Ts. Hence, the Th or Ts is triggered by antigen in the context of the appropriate

MHC-related determinant, while the recognition site for the MHC-related determinant on the antigen-binding chain explains why the antigen-nonbinding chain is I-A$^+$ in ThF and I-J$^+$ in TsF. The recognition site for MHC-related determinants or the MHC determinant itself also accounts for the genetic restriction in the action of these factors (see Figure 1 and the next section).

The evidence for a genetic restriction in interchain complementation is summarized here. In the case of ThF, the separated chains show a genetic restriction in their complementation that maps to I-A (38). In general a genetic restriction implies both a polymorphic determinant, e.g. I-A, and a recognition site for that determinant; this basic rule allows the deduction that one chain bears I-A determinant(s), as is already known from serological studies, while the other chain bears a recognition site for I-A. The data on the genetic restriction in interchain complementation for TsF are incomplete. In the case of picryl (41), NP (2), and GT (34) TsF-eff, the two chains separated from the disulfide-bonded molecule show a genetic restriction. This has been studied using the naturally occurring free chains in the picryl system and maps to the I-J region (41). This interaction between the chains may be a preliminary to intracellular disulfide bonding.

Comparison Between Secreted T-Cell Factors and the T-Cell Receptor

Inspection of Figure 1 shows that the antigen-binding chain of ThF, like the T-cell receptor, has recognition site(s) for antigen and I-A. This suggests that the antigen-binding chain of the ThF uses the same gene rearrangements as the T-cell receptor of the cell that made it. The question then arises as to whether the two chains of ThF correspond to the α and β chains of the T-cell receptor? The current view is that the T-cell receptor of the helper is a heterodimer ($\sim$80 kd) composed of an α and a β chain, both involved in antigen recognition, that apparently lack I-A and presumably I-aT (56) determinants by biochemical criteria. Similarly, the T-cell receptor of a Ts hybridoma (28 kd) lacks covalently linked I-J determinants (57). In this respect the α and β chains of the T-cell receptor differ from the antigen-nonbinding chain of the secreted products that bears MHC-related determinants. Moreover, it is unlikely that the antigen-binding chain corresponds to both the α and β chains, as there is no clear evidence that it can be dissociated into two different chains. Further polyacrylamide gel studies are needed.

This simple model, in which the recognition site for interchain complementation uses the same gene rearrangements as the receptor on the T cell that makes it, is a modern rephrasing of Ehrlich's side-chain theory. This analogy predicts that the cell that makes ThF, like the ThF itself,

should recognize I-A, and in keeping with this, picrylated cells injected into the footpad only induce ThF when they are I-A matched with the recipient (38). Similarly the cell that makes TsF, like the TsF itself, should recognize I-J, and in keeping with this, only haptenized cells of the appropriate I-J genotype induce Ts when injected intravenously (2, 23, 24).

The reason that ThF induces positive "helper" and not suppressor responses is probably that ThF bears I-A determinants and hence can favor the presentation of antigen in the context of I-A. A similar argument may explain why the lysozyme TsF-ind, which is I-J restricted, induces second-order suppressor and not helper T cells. Whether Ts-eff and nonspecific TsF can also induce further suppressor cells by virtue of their I-J determinants is unclear.

The alternative view is that there are two separate MHC recognition sites on the antigen-binding chain that is analogous to the T-cell receptor—one site for MHC-restricted antigen recognition and the other for MHC-restricted interchain complementation. There is formal evidence supporting this view in the case of TsF. (See section on Crosslinking and I-J Are Required To Trigger the T-Acceptor Cell.) This model with two-MHC recognition sites allows the possibility that the two sites may show different MHC specificities (crossed systems model). In this situation, an antigen recognized in the context of one determinant, e.g. I-A, might stimulate the production of a factor bearing a different determinant, e.g. I-J. Hence, an antigen seen in a context normally giving rise to help might cause suppression.

Mode of Action of Antigen-Specific T-Cell Factors

The I-A determinant(s) and the recognition site(s) for I-A on ThF suggest several ways in which it may act as a helper factor, and similar arguments apply for Ts-ind, which bears I-J determinants and presumably has recognition site(s) for I-J. In fact ThF may:

1. *Provide I-A*, which together with antigen triggers the T cell. Both this and Mechanism 2, below, would account for the intriguing observation that there is an I-A genetic restriction in the action of ThF, i.e. picrylated allogeneic cells induce contact sensitivity, providing they are incubated in ThF that is I-A compatible with the recipient (38).

The analogy for TsF-ind is the provision of I-J, which together with antigen triggers the Ts. This and Mechanism 2 below predict that the induction by Ts-ind is genetically restricted by the MHC locus and in particular the I-J region. This is seen in the lysozyme (7) and picryl (25) systems in which TsF-ind together with antigen gives rise to Ts-eff. However, this is unlikely to be the sole mechanism in systems lacking this genetic restriction. These include the GAT system (34) and systems in which

Ts-ind induces idiotypic Ts-trans. These considerations do not explain why the TsF-ind and TsF-eff, in the lysozyme system, have the same fine specificity for the suppressor epitope. However, this may be due to a disproportionate representation of T cells directed to the suppressor epitope or to an idiotype–anti-idiotype loop.

2. *Attach to I-A$^+$ antigen-presenting cells.* After ThF attaches to I-A$^+$ cells through its recognition site(s) for I-A, the antigen-binding site may serve to concentrate antigen. Although macrophages and dendritic cells are the obvious I-A$^+$ antigen-presenting cells, there may also be I-A$^+$ antigen-presenting T cells that produce or passively acquire I-A determinants. Similarly, TsF-ind may bind to an I-J$^+$ macrophage or T cell through its recognition site(s) for I-J.

3. *Attach to antigen-presenting cells by mechanisms not involving recognition of I-A.* ThF may be cytophilic for certain antigen-presenting cells and, hence, may concentrate antigen on their surface and perhaps increase the amount of I-A available to facilitate the interaction of antigen with T cells.

On the suppressor side, this mechanism is relevant for those TsF-ind that are not genetically restricted in their action and, hence, cannot act only as a source of I-J or receptor for I-J. In these situations the antigen-presenting cell provides I-J, since there is an I-J genetic restriction between the antigen-presenting cell (macrophage) and the recipient. This is seen in the NP (2), ABA (12), and TMA (33) systems in which the TsF-ind provides both help and antigen (idiotype) and generates anti-idiotypic Ts-trans.

4. *Arm a cell that then releases nonspecific factors when exposed to antigen.* This possibility is formally analogous to the action of Ts-eff through a T-acceptor cell or macrophage (19).

5. *Interfere with suppressor-cell responses.* This is a possible mode of action of ThF formally analogous to contrasuppression (1). For instance, some evidence suggests that certain I-J$^+$ molecules are receptors for self I-A (56). The implication is that I-A and I-J molecules may interact and hence I-A$^+$ ThF might interfere with I-J$^+$ Ts. Similarly I-J$^+$ TsF might interfere with I-A-based immune responses.

BIOLOGICAL CONSIDERATIONS It is not clear whether ThF is essential for the induction of contact sensitivity or only increases its magnitude, nor whether TsF-ind is essential for the Ts-eff response or only increases its size. In any event there are two different effects of Ts-ind: the induction of idiotype-directed Ts-trans and of antigen-directed Ts-eff. No system is known in which the same TsF-ind gives rise to both Ts-trans and Ts-eff (but see 33). It may be that these effects are due to two different classes of Ts-ind. Alternatively, the basic rule may be that Ts-ind alone gives rise to Ts-trans, and Ts-ind together with antigen gives rise to Ts-eff, and the exceptions may

have special explanations. Thus, in the TMA system antigen together with TsF-ind is required to produce the anti-idiotypic Ts-trans. Perhaps anti-idiotypic cells are so poorly represented in the repertoire that their number must be boosted by idiotype-positive cells induced by antigen. Other alternatives are that antibody induced by the antigen provides the idiotype or that the idiotypic determinant recognized by the Ts-trans is only generated when the idiotype combines with antigen.

Although TsF-ind induces antigen-directed Ts-eff when injected with antigen, it fails to induce idiotype-directed Ts-trans when injected alone; this may be due to the use of monoclonal Ts-ind in a system that lacks a dominant idiotype (7). Under these conditions, any Ts-trans produced would only interact with the idiotype of a minority of the Ts-eff and might escape detection.

ANTIGEN-SPECIFIC T-SUPPRESSOR FACTORS

Molecular Biology of T-Suppressor Factor

Although biologically active antigen-binding and I-J$^+$ chains of TsF can be produced by translating mRNA (58, 59), there are only limited data about the molecular biology of T-suppressor cells. Radiation leukemia virus which make antigen-specific TsF-ind show rearrangement of clones α, β, and γ genes of the T-cell receptor. The β gene was expressed in both a lysozyme-specific and an acetylcholine-receptor-specific clone. However, the lysozyme clone failed to express α or γ mRNA, while the other clone expressed comparable amounts of β and γ and a small amount of α (60). β gene rearrangement has also been found in a human line (OKT4) that suppresses antibody (61). The deletion of the area of the β gene in the DNA and the failure of β expression in Ts hybridomas were unexpected. It is possible that only a minority of the cells were producing TsF or had Ts cell receptors (62).

Molecular Forms of Antigen-Specific T-Suppressor Factor

The production of TsF-eff, which blocks the passive transfer of contact sensitivity, requires both injection of picrylsulfonic acid and reexposure to antigen by painting (4, 66). This is because the injection of PSA leads to the production of the antigen-binding chain of TsF that is inactive alone, while the final painting with picryl chloride causes the production of an additional I-J$^+$ chain. In fact, the TsF activity that inhibits the passive transfer of contact sensitivity can arise in two different ways. It may be due to a two-chain, I-J$^+$ disulfide-bonded molecule around 90 kd (46). Alternatively, it may be due to complementation between two separate molecules, one antigen-binding and the other bearing I-J determinants,

which may arise from a single cell (40) or from distinct cells of different phenotypes (41; see also 49).

HIGH-MOLECULAR-WEIGHT, DISULFIDE-BONDED HETERODIMER The culture supernatants of lymphoid cells from mice injected with PSA and then painted with picryl chloride contain TsF activity around 90 kd, due to disulfide-bonded molecules. This TsF dissociates on reduction and alkylation into an antigen-binding I-J$^-$ chain and I-J$^+$ antigen-nonbinding chain. Both chains are required for biological activity. The I-J genetic restriction in this interchain complementation was studied using naturally occurring separate chains. It implies that one chain is I-J$^+$, a fact already known from serological studies, while the other chain has receptors for I-J (40). The presumption is that the two chains are both made and disulfide bonded within a single cell; the production of disulfide-bonded TsF by hybridomas supports this view (2, 51, 52).

SEPARATE ANTIGEN-BINDING AND I-J$^+$ CHAINS The same supernatant also shows activity around 50 kd, due to separate antigen-binding and I-J$^+$ chains as shown by affinity chromatography on antigen or anti-I-J. The presumption is that there is a loose complex of antigen-binding and I-J$^+$ chains or that both chains occur separately, perhaps as homodimers, with a M_r of $\sim$50 kd. Finally, the supernatants contain separate antigen-binding and I-J$^+$ chains with M_r of $\sim$35 kd and $\sim$25 kd, respectively, by gel filtration (40).

As the two chains of TsF are both required for biological activity, they are important assay reagents for each other. In fact, the antigen-binding chain can be obtained relatively free from the other chain by immunochemical or biological methods. These include:

1. Making culture supernatants containing both disulfide-bonded and free chains with cells from mice injected with PSA and then painted with picryl chloride, and then removing the disulfide-bonded molecules from the supernatants, by absorption with anti-I-J antibody.
2. Splitting crude culture supernatant or high molecular weight TsF with dithioerythritol and then reducing and isolating the antigen-binding chain by affinity chromatography followed by elution with 3 M KCNS, 0.1 M NH_4OH, or "picryl-ε-aminocaproic acid."
3. Using culture supernatants that lack appreciable amounts of disulfide-bonded TsF or I-J$^+$ chains. These are regularly produced in our laboratory by injecting PSA without subsequent painting (43; but see 49). In this case, activity is found around 35 kd and 70 kd by gel filtration.

The I-J$^+$ chain can be obtained by a variation of method 1 or 2 above. It also occurs in a potassium chloride extract of normal spleen cells. (The KCl

probably acts by activating proteolytic enzymes, which release surface I-J, and by breaking cells, which releases cytoplasmic I-J.) I-J also occurs in the 24-hr culture supernatant of haptenized, but not of normal, spleen cells. It is not clear whether haptenization acts by killing cells or by activating synthesis, secretion, or shedding.

TSF ACTIVITY MAY BE DUE TO THE PRODUCT OF A SINGLE CELL OR TWO DISTINCT CELLS In summary, TsF can appear as a disulfide-bonded molecule or as two separate chains made by a single cell. Alternatively, the two chains may be made by distinct cells. Thus, Ly-1$^-$2$^+$ I-J$^+$ cells from mice injected with PSA make the antigen-binding chain, while haptenized Ly-1$^+$2$^-$ I-J$^+$ normal spleen cells make the I-J$^+$ chain (41). Similar observations have been made in the sheep red blood cell system which suppresses antibody production (55).

DISTAL COMMON PATHWAYS IN THE T-SUPPRESSOR EFFERENT CIRCUIT

Concept of the Nonspecific Acceptor Cell

Although TsF-eff may have a direct action on Th or B cells, it has no direct effect on the cells that mediate contact sensitivity and cannot affect passive transfer when the cells are taken from donors which have received cyclophosphamide. Its nonspecific mode of action can be readily understood through its analogy with the IgE/mast cell system. Figure 2 illustrates

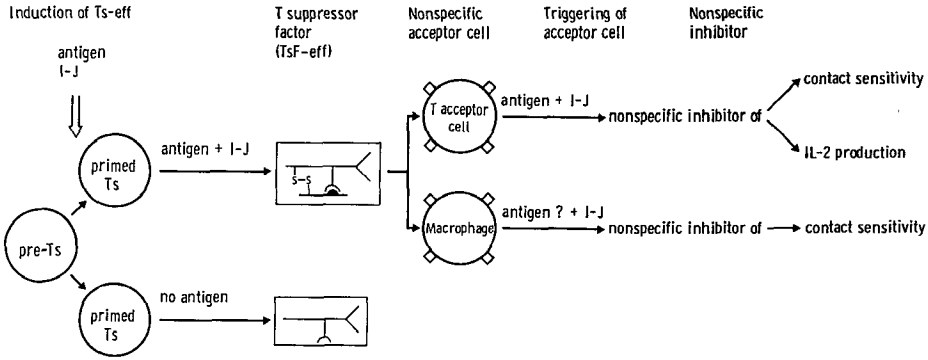

Figure 2 T suppressor efferent/T acceptor cell circuit. The diagram illustrates that the initial immunization is by antigen together with I-J. In some circuits this may require help from Ts-ind. The Ts-eff that results from this immunization secretes the antigen-binding chain of TsF-eff. However, it releases high molecular weight, two-chain, disulfide-bonded TsF-eff when stimulated with antigen and I-J. (In the NP system an idiotype-directed Ts-trans also triggers the secretion of TsF by the Ts-eff.) The TsF arms an acceptor cell, the T-acceptor cell, or macrophage. The T-acceptor cell is then triggered by antigen and I-J to release nonspecific TsF, which inhibits contact sensitivity and the production of IL-2.

that TsF arms the T-acceptor cell or macrophage. These may be regarded as nonspecific *acceptor cells* that lack intrinsic *relevant* immunological specificity but acquire it when armed with antigen-specific TsF-eff. Triggering with antigen and, at least in the case of the T-acc, with I-J liberates nonspecific inhibitors. The specificity of the antigen and the genotype of the I-J must correspond to the TsF-eff and not to the acceptor cell. This is an amplification system and enables a small amount of specific factor to cause the release of a large amount of a nonspecific factor in an antigen-specific fashion.

The Macrophage as an Acceptor Cell

It may be useful to trace the development of the concept of the nonspecific acceptor cell. Early studies showed that macrophages absorb TsF and are then able to inhibit the passive transfer of contact sensitivity (63, 64). In fact, the armed macrophages release a nonspecific macrophage suppressor factor (M_r 10–20 kd) when exposed to picrylated cells or picrylated protein (18, 65). It is not clear whether both antigen and I-J are required for this release. The factor inhibits the passive transfer of contact sensitivity. There is some evidence that it also inhibits cell proliferation in vitro (66).

This macrophage suppressor factor may play a role in suppression of the response to NP. In this system the anti-idiotypic TsF-trans can be absorbed by adherent cells (macrophages) and still cause suppression. There is an I-J genetic restriction between the macrophage and the TsF-trans, but not between the TsF and final recipient (15). One possible explanation of this genetic restriction is that the macrophage armed with TsF-trans is triggered by the combination of idiotype in the final recipient and the I-J of the macrophage to release nonspecific macrophage suppressor factor.

T-Acceptor Cell

The ability of TsF-eff to attach to immune cells in the cold and its failure to inhibit without incubation at 37°C suggested an indirect mode of action (18). In the DNP contact sensitivity system, a T-suppressor auxiliary cell is required for the action of Ts cells produced by painting with supraoptimal doses of antigen (32). In fact, a similar cell is required for the action of antigen-specific TsF-eff in the picryl and oxazolone systems. Thus, TsF-eff does not affect passive transfer by cells from mice subjected to adult thymectomy or cyclophosphamide. However, the inhibition can be restored by adding 4-day immune cells produced by the same or an unrelated antigen. Analysis of this system led to the recognition of the nonspecific T-acceptor cell with the following properties (17, 19):

1. The cell is produced by painting the skin with contact sensitizing agents,

but specific immunization is not required. The cell is not found in resting mice but appears by 2 days after immunization in the lymph nodes and spleen and disappears by day 10. Similar cells are probably produced by injecting haptenized cells in the footpad and may also occur 2 weeks after immunization with antigen in Freund's complete antigen (67). The T-acc also occur in mice given large doses of BCG intravenously (see 66).

2. The cell is Thy-1^+, Ly-1^-2^+, FcR^+, I-J^+.
3. The cell is sensitive to adult thymectomy and to cyclophosphamide given before immunization. This effect may be on antigen-presenting cells (23) or on the precursors of the T-acc.
4. The cell possesses receptors for TsF-eff. Thus, T-acc adhere to haptenized albumin on a plate when armed with the appropriate TsF-eff. This provides formal proof that TsF binds to the surface of the T-acc.
5. The cell is triggered to release nonspecific inhibitor by antigen together with I-J.

Similar T-acc are required in the TMA antibody (67), lysozyme delayed hypersensitivity (7) and NP delayed hypersensitivity systems (27, 28); and at least in the last two, the T-acc do not require specific immunization for their production. The contrasuppressor circuit in the picryl system also utilizes an I-J^+, cyclophosphamide-sensitive cell that requires immunization, but not specific immunization, for its production; it is similar or possibly identical to the T-acc. This cell liberates a nonspecific contrasuppressor factor when armed with antigen-specific contrasuppressor factor and then triggered with antigen (68).

COMMENTS ON I-J Although this article is not primarily about I-J, a brief comment may be helpful. This subject has been recently reviewed (see 56). I-J was originally regarded as the product of an MHC locus lying between A and E. This was not confirmed by DNA studies, and a locus on chromosome 4 may also be involved (69). This led to the suggestion that I-J was, or shared determinants with, a receptor for recognizing self-MHC (56). This is based on the finding that certain anti-I-J sera react with responder cells and inhibit the mixed lymphocyte reaction. I-J determinants may also be associated with E_β, as both these determinants occur on the antigen-nonbinding chain of the TsF in the LDH system (16). In any event, both conventional and monoclonal antibodies recognize different I-J determinants on Ts-ind and Ts-eff (70); several authors have pointed out that anti-I-J sera might recognize receptors for I-J, because the congenic mice used to produce them differ in both I-J and recognition molecules for I-J. Nevertheless, the term I-J is still useful for the loci that distinguish B10.A(3R) and B10.A(5R) mice and for the products recognized by anti-I-J sera.

CROSSLINKING AND I-J ARE REQUIRED TO TRIGGER THE T-ACCEPTOR CELL The triggering of the T-acc requires both antigen and I-J corresponding to the source of the TsF and not to the source of the T-acc (17, 20, 26). This was shown using picrylated cells from B10.A(3R) and B10.A(5R) mice as a source of antigen. This genetic restriction is usually attributed to an I-J recognition site on the TsF that interacts with I-J on the haptenized cell, and not to the I-J of the TsF interacting with an I-J recognition site on the haptenized cell. This I-J genetic restriction in triggering is in addition to the I-J restriction in the complementation between the two chains of the Ts-eff.

In fact, the antigen-binding chain of TsF has two recognition sites for I-J: one for interchain complementation and the other for triggering, when the TsF is attached to the surface of the T-acc. This view is based on studies in (CBA × B10)F1, i.e. (k × b)F1, mice. These mice produce distinct I-J^k and I-J^b disulfide-bonded TsF. Two separate antigen-binding chains are obtained by affinity chromatography on anti-I-J columns, followed by reduction and alkylation: one is only complemented by an I-J^k antigen-nonbinding chain and the other by an I-J^b. The key observation relates to the genetic restriction in the triggering of the T-acc armed with antigen-binding chain of F1 origin complemented with the corresponding antigen-nonbinding (I-J^+) chain of parental origin. Under these conditions, either H-2^k or H-2^b picrylated cells can be used as a source of antigen for triggering. However, there is still a genetic restriction, as picrylated third-party cells (H-2^d) are ineffective. In other words, antigen-binding chain derived from I-J^k-bearing TsF of F1 origin (but not of parental origin) complemented with I-J^k chain (of F1 or parental origin) recognizes antigen in the context of either I-J^k or I-J^b at the triggering stage. Whether this is due to two types of antigen-binding chains, one that recognizes antigen in the context of I-J^k and the other in the context of I-J^b, or to a single type of chain that recognizes both I-J^k and I-J^b is unclear. In any event, the fact that the genetic restriction for interchain complementation can be different from the genetic restriction for triggering implies that there are two distinct I-J recognition sites on the antigen-binding chain, one for interchain complementation and the other for triggering (unpublished data, M. Zembala).

Subsequent experiments showed that there was no need for the hapten and the I-J to be on the same "antigen-presenting" cell, e.g. T-acc armed with TsF of genotype I-J^k could be triggered by picrylated I-J^b cells providing I-J^k cells were added (M. Zembala, unpublished data). This suggests that antigen or I-J or both could move to the surface of the armed T-acc. Attempts to simplify the source of the antigen and the I-J showed that antigen could be provided by picrylated albumin or by the bivalent,

low-molecular-weight hapten, bis(picryl)-L-lysine, while the I-J could be provided by normal cells or by extracts of normal cells. Affinity-purified I-J can also be used (26).

Further analysis was based on studying bifunctional, mixed haptens in which the dibasic amino acid lysine was derivatized on the α-amino group with picryl (or NP) and on the terminal ε-amino group with oxazolone. This gave a molecule univalent with respect to either hapten but able to crosslink TsF of picryl and oxazolone specificity with each other. Experiment showed that these mixed haptens triggered T-acc armed with both TsF, but not cells armed with only one factor. This implies that crosslinking of separate molecules of TsF leads to triggering, as these mixed haptens are unable to crosslink the combining sites of a single molecule. Occupancy and perhaps crosslinking of the recognition site for I-J is also required. It is interesting that the I-J and the antigen can be given in any order, which makes it likely that the receptor has sufficient affinity to bind the hapten and the I-J alone (26). It would be interesting to know whether there is a positive cooperativity of binding so that binding of antigen or I-J increases the affinity of binding of the other factor, as suggested in a recent model of the T-cell receptor (44).

EQUIVALENCE OF CONVENTIONAL AND MONOCLONAL TsF-EFF This collaboration between TsF of different antigen specificities indicates that oxazolone and picryl Ts-eff belong to the same class. Similar experiments show that monoclonal NP TsF_3 is functionally equivalent to oxazolone TsF-eff. In particular, T-acc armed with a mixture of conventional oxazolone TsF-eff and monoclonal NP TsF-eff (TsF_3) are triggered by the mixed hapten "NP-oxazolone-lysine." Further evidence for the equivalence of these two factors is their common action through the nonspecific T-acc and the I-J genetic restriction in triggering (27).

Nonspecific T-Suppressor Factor That Inhibits the Transfer of Contact Sensitivity

The nonspecific-acceptor cells, the macrophage and the T-acc, and their nonspecific suppressor factors provide a common pathway for many Ts-eff. In fact, the acceptor cell system provides an example of the specific activation of a nonspecific effect. In these systems, the initial antigen-specific TsF-eff gives rise to a nonspecific factor in the presence of the corresponding antigen and I-J. Hence, the usual specificity control of asking whether antigen-specific TsF-eff affects a third-party response cannot show a nonspecific effect unless antigen and I-J corresponding to the TsF are also present (see 64). Despite this final nonspecific effect, it is

likely that the distinctive location of antigen and movement of cells to the local environment limit generalized nonspecific suppression in the intact animal (71).

The inhibitory factor(s) made by T-acc (nonspecific TsF) has two main effects—the inhibition of the passive transfer of contact sensitivity and the inhibition of IL-2 production. The inhibition of the passive transfer of contact sensitivity is due to a molecule of $M_r \sim 50$ kd by gel filtration and a pI around 6.7. It is antigen nonspecific and MHC unrestricted. The factor itself is I-J$^+$. However, it is made by the T-acc, and the I-J is not derived from the antigen-specific TsF, as the phenotype of the I-J corresponds to the T-acc and not to the TsF (50). The factor may act by blocking lymphokine production—a possibility suggested by the finding that antigen-specific TsF-eff, which can act through nonspecific TsF, blocks the production of an inhibitor of the migration of T cells (72). The interpretation of experiments, which suggest that the present nonspecific TsF can be produced in the absence of T-acc, may be complicated by the presence of macrophages acting as acceptor cells, since the T cells were not purified. Alternatively the depletion of T-acc cells may have been incomplete (73).

Nonspecific T-Suppressor Factor That Inhibits the Production of IL-2

The supernatants that block the transfer of contact sensitivity also block the Concanavalin A (Con A)–induced production of IL-2 when added within the first 8 hr of culture. This is not a toxic effect, as nonspecific TsF has no effect when added before stimulation with Con A and then washed off (20, 21). Analogy with a human factor produced by cells stimulated with *Candida albicans* in vitro (74) suggests that nonspecific TsF may act by blocking IL-1 production by macrophages (74).

The nonspecific TsF is absorbed by adherent cells (macrophages) and by activated, but not resting, lymphocytes (M. Malkovsky, personal communication). It does not act on the cell responding to IL-2; IL-2 shows the same activity in stimulating Con A blast cells whether diluted in medium or in nonspecific inhibitor. This apparently distinguishes it from the factors described in serum of normal mice and mice infected with malaria (75, 76). However, interpretation may be complicated as inhibition of IL-1 production may interfere with the appearance of receptors for IL-2.

This factor is probably different from stimulated rat T cell–derived inhibitory factor (STIF), which blocks the response to IL-2 (77); from soluble immune response suppressor (SIRS), which does not affect T-cell proliferation; from inhibitor of DNA synthesis (IDS), which has a pI of ~ 2.9 (see 78); and from a factor produced by cells in the mixed lymphocyte reaction that inhibits the production of IL-2 (79).

In keeping with its effect on the production of IL-2, nonspecific TsF limits Con A–induced DNA synthesis in vitro. It also limits antigen-induced lymph node proliferation and contact sensitivity in vivo when given at the time of immunization. The pI and molecular weight of the factor that inhibits the transfer of contact sensitivity and DNA synthesis in vivo are comparable when tested in parallel. Moreover, the pI of the nonspecific T-suppressor factor(s) that blocks IL-2 production and the transfer of contact sensitivity are similar (50). Nevertheless, further studies are needed to determine whether the T-acc makes a single nonspecific or a family of nonspecific TsF.

Control of the Balance Between Immunity and Suppression

In general, antigen + I-J is the cue for inducing Ts (2, 23, 24), while antigen + I-A induces Th responses. However, it is unlikely that this is the only determinant of the relative activity of helper and suppressor circuits. One possibility is that the level of production of IL-2 is an important determinant. For instance, if Ts require less IL-2 than Th (or have a higher affinity for IL-2) then the suppressor circuits would dominate when IL-2 is rate limiting, while helper circuits would dominate if more IL-2 is present. The following experiment suggests that IL-2 may indeed influence the balance between unresponsiveness and immunity. Normally the intravenous injection of picrylated cells gives rise to unresponsiveness associated with suppressor cells. However, contact sensitivity occurs when IL-2 (or Con A that induces the formation of IL-2) is injected at the same time (80, 80a). This shows that exogenous IL-2 influences the balance between immunity and unresponsiveness and raises the question whether endogenous IL-2 has the same effect. These considerations may elucidate the contrast between the induction of unresponsiveness by antigen given intravenously and the immunity by antigen given subcutaneously (81). This might be due to differences in the amount of IL-2 produced and the amount of IL-2 needed to initiate immune responses in the spleen as compared with those in the lymph nodes. Further analysis requires separate study of unresponsiveness associated with suppressor cells and unresponsiveness in which suppressor cells cannot be demonstrated; these types of unresponsiveness differ in the MHC requirements for their induction and in their sensitivity to cyclophosphamide (82, 83).

Further evidence of the importance of IL-2 in influencing the outcome of the immune response has been obtained in transplantation systems. In neonatal tolerance, using the (CBA × B10)F1 → CBA combination, in which transplantation tolerance is difficult to induce, IL-2 prevents the induction of tolerance when given 1 day after the injection of the

semiallogeneic cells used to induce tolerance (84). One possible mechanism is that the IL-2 stimulates an immune response that eliminates the injected cells whose presence is needed for the persistence of tolerance. Moreover IL-2 slowly terminates neonatally induced tolerance in mice bearing a tolerated skin graft over about a month. The implication is that limitation of IL-2 production is important for the maintenance of tolerance in this system (85).

The following experiment suggests a possible link between Ts and the clonal anergy that is seen after the injection of PSA, as assessed by limiting dilution studies of cytotoxic cell precursors (86). In preliminary experiments a primary mixed lymphocyte reaction (CBA anti-B10) was conducted in the relative absence of IL-2 produced by nonspecific TsF that depresses IL-2 production. At 4 days, the viable cells were isolated and restimulated with the same antigen (B10) or third-party antigen (B10.S). The relative lack of IL-2 in the primary led to selective loss of the secondary mixed lymphocyte reaction with retention of the third-party response. Mixture experiments showed no evidence of suppressor cells (M. Malkovsky, unpublished data). These results raise the possibility that the Ts-eff/T-acc circuit, which produces a nonspecific TsF that inhibits the production of IL-2, may be one of the mechanisms of clonal inactivation of T cells (see 87).

Another level of control of unresponsiveness is suggested by the finding that agents that selectively derivatize SH groups cause suppression, while those that derivatize amino groups cause immunization when applied to the skin (88, 89). Although these differences may be due to effects on the distribution or persistence of antigen, or to a requirement for amino derivatization for activating positive responses, it is possible that there are free SH groups on I-J or molecules that associate with I-J and hence that selective SH (thiol) reagents preferentially activate Ts.

The Role of the T-Acceptor Cell in the Depression of Contact Sensitivity by Antigen (Desensitization In Vitro), T-Suppressor Factors, and Anti-Idiotypic Antibody

The Ts-eff/T-acc cell circuit offers a simple explanation of the ability of antigen, TsF-eff, anti-idiotypic TsF-trans, and anti-idiotypic antibody to suppress the passive transfer of contact sensitivity. In the picryl system 4-day immune cells transfer contact sensitivity. However, this is blocked by incubation for 1 hr with antigen-specific TsF or syngeneic picrylated cells.

Why does either antigen or TsF suppress passive transfer? The likely answer is based on the presence of antigen on the surface of lymph node cells, probably T cells and macrophages, 4 days after immunization, as shown by the cells' ability to immunize other mice (see 90). By adding a large

amount of TsF, the small amount of antigen available is sufficient to trigger the release of nonspecific TsF from the T-acc. Alternatively, when a large amount of antigen (haptenized cells) is added, the release of TsF may be stimulated and this then arms the T-acc, which is then triggered by the antigen to release nonspecific TsF. In keeping with this interpretation, desensitization in vitro requires the presence of two cells: one is the antigen-specific Ts-eff, which is cyclophosphamide resistant, adult-thymectomy sensitive, and antigen adhering; the other is nonspecific T-acc, which is sensitive to both cyclophosphamide (91) and adult thymectomy and adheres to antigen only after arming with TsF (92). (See Figure 2.)

In the NP system, mice are routinely immunized by injecting NP-modified cells subcutaneously. These mice show delayed sensitivity but also have Ts-eff and probably T-acc (28, 67) in their lymph nodes. Their delayed sensitivity can be inhibited by the injection of anti-idiotypic TsF-trans (TsF_2), which is known to trigger the release of TsF-eff (TsF_3) even in the absence of antigen. The presumption is that the anti-idiotypic factor is an internal image of antigen, and the situation is formally analogous to desensitization in vitro. As the factor is I-J$^+$, it is possible that it provides the I-J determinants necessary to trigger the Ts-eff to make TsF-eff (but see section on *The Macrophage as an Acceptor Cell*). Anti-idiotypic B cells (93) and anti-idiotypic antibody (94) that affect contact sensitivity probably act through the T-acc (95).

T-Suppressor Afferent Cells That Do Not Act Through the Ts-eff/T-acc Circuit

Some Ts that only suppress when given early in the immune response do not act by inducing Ts-eff or anti-idiotypic Ts-trans of the type already discussed. The argument goes as follows: The Ts-aff that affect contact sensitivity and DNA synthesis in vivo cannot be Ts-eff, because they only act when given early and show different sensitivities to adult thymectomy and cyclophosphamide. Moreover, they cannot have an obligatory mode of action through the Ts-eff/T-acc circuit, as they are active when injected into mice that cannot generate these cells as a result of adult thymectomy (5, 35, 36). The properties of the Ts-aff are summarized below (5):

1. Ts-aff are produced by giving two injections of PSA or by painting with picryl chloride. In the dinitrophenyl system they are also produced by injecting haptenized lymphoid cells or cells taken from mice that contain Ts-eff (and presumably other cells) (31). The ability to produce these Ts-aff after adult thymectomy shows that Ts-eff are not necessary for their induction, but it is an open question whether the Ts-eff is one of the cells that can facilitate their induction (31).

2. Ts-aff cannot be produced in mice given cyclophosphamide before

immunization or infected with Newcastle disease virus (96), but their generation is unaffected by adult thymectomy. The target for the action of cyclophosphamide may be the precursors of the Ts-aff or the antigen-presenting cell needed for its generation (23). In contrast, the Ts-eff has the opposite sensitivities and cannot be produced after adult thymectomy, but is unaffected by Newcastle disease virus or cyclophosphamide given before immunization. The T-acc is sensitive to all three procedures. When cyclophosphamide sensitivity is studied one day after immunization, it is unclear whether the generation of the Ts-eff and/or the T-acc is affected (2, 28).

3. Ts-aff specifically block the secondary IgG but not the IgE response to picryl chloride (97, 98); they also block the in vivo proliferation in lymph nodes following immunization (36, 99) and affect the induction of cytotoxic cells (100). In the GAT system, Ts-aff inhibit delayed hypersensitivity but not proliferation. This might suggest that there are two distinct Ts-aff. Alternatively a single Ts-aff might carry out both activities, but one is blocked by another cell (101). It is not clear whether all these effects are secondary to inhibition of the production of antigen-specific ThF, which augments contact sensitivity (102), or whether there is a family of closely related Ts-aff.

4. These cells also affect the induction of contact sensitivity, perhaps by blocking the production of ThF. However, they cannot be demonstrated in mice painted with the standard dose of picryl chloride used to produce contact sensitivity, perhaps because of the presence of T-helper and contrasuppressor cells in the immune population (68).

It is likely that the Ts-aff produced by injecting haptenized cells, which affect DNA synthesis and occur in the absence of Ts-eff, belong to the same family and in this case are probably anti-idiotypic (31). There are numerous other Ts that affect antibody production and delayed hypersensitivity and in which a Ts-eff/T-acc circuit has not been described; these Ts may also belong to this family (103, 104). However, detailed studies are needed because the Ts-eff/T-acc circuit, which leads to the production of a nonspecific TsF that blocks the production of IL-2 and perhaps other lymphokines, is also able to suppress delayed and contact sensitivity and may in principle affect cell proliferation.

Biological Function of T-Suppressor Cells

Why is there a need for both suppressor-inducer and suppressor-effector cells? This is analogous to the general question of why T-T and T-B collaboration is required. The classical answer is that two-cell systems lend themselves to greater amplification and finer control. Perhaps the alternative—a cell that provides help for itself, e.g. a Ts-inducer cell that is

also a Ts-effector cell—is too difficult to control. An additional consideration is that the animal needs a memory of the antigen at the time of the initial invasion by the microorganism. For instance, viruses must have the property of being able to adhere to cells so that they can enter them; the molecules used at that time—and perhaps the molecules with which they react—are the best target for the main immune response. However, several days after infection, the production of free viral-coat protein might cause the immune system to give a response appropriate to a large amount of soluble protein. Perhaps the T-helper (inducer) cell in the two-cell system provides a memory for the initial mode of presentation of antigen and prevents the response being unduly influenced by the large amount of viral protein produced later. This argument applies equally to conventional and suppressor cell responses.

What is the role of anti-idiotypic transducer cell in suppressor circuits and of the anti-idiotypic helper cell in conventional systems? One possibility is that they provide a method of anticipating antigens arising from antigenic variation in microorganisms. One method is the mutation of variable-region genes. An alternative is an idiotype anti-idiotype loop that induces further cells, which share an idiotype with the original immune response but are not necessarily directed to the same antigen. This may increase the number of cells that have a greater than random chance of reacting with new antigens arising from antigenic variation.

Another possibility emerges from the ability of various antibodies, and perhaps T-cell products, to enter the fetus. Anti-idiotypic factors may affect the clonal distribution of cells in the offspring and hence provide an additional method whereby the antigenic experience of the mother may influence the child. The basic consideration is that the microorganisms met by the mother are a guide to those likely to be met by the offspring.

It is interesting that the amount of microorganismal antigen rises following infection. In general, successful handling of the infection leads to a decline in the rate of rise of the amount of antigen and later to an absolute decline, while failure to control the infection leads to a progressive rise in the amount of antigen. In the first situation, an orderly reduction in the size of the immune responses is required that makes provision for recrudescence of the infection and antigenic variation. In the second situation, there are three rational strategies: (*a*) augmentation of the ongoing response, which may be qualitatively appropriate but too small; (*b*) changing the nature of the ongoing responses which may be inappropriate for the particular organism; and (*c*) diminution at least of those responses that cause immune damage. Clearly there is no general purpose "correct answer," and the need to prevent a whole species from being killed by a variant microorganism requires that responses to different antigens and modes of presen-

tation of antigen should be subject to extensive genetic and environmental variability within a species. Perhaps we are studying a system in which variability of results between different members of the same species is an essential feature.

Summary

This review gives an overview of two main suppressor circuits. In its complete form, the first circuit form has an early acting Ts-inducer that behaves like a T-helper cell for the production of idiotype-directed Ts-transducer or antigen-directed Ts-effector cells. In this circuit, the T-suppressor effector cell (Ts-eff) produces antigen-specific T-suppressor factor (TsF). This has a mode of action through the T-acceptor cell (T-acc), a cell that requires immunization, but not specific immunization, for its production. This cell, when armed with TsF-eff and then triggered with antigen and I-J, releases nonspecific TsF that blocks the passive transfer of contact sensitivity. It also blocks the production of IL-2. The biological significance of the complexities of this circuit is discussed in relation to the control of unresponsiveness and the handling infection and antigenic variation of microorganisms.

The second set of suppressor cells, T suppressor afferent cells, inhibits only when given early in the immune response but differs from the Ts-inducer by lacking an obligatory mode of action through the Ts-eff/T-acc circuit.

In general, the antigen-specific T-helper and T-suppressor factors have a two-chain disulfide-bonded structure. One chain carries the antigen-binding site and the other chain MHC-related determinants. Both chains are required for biological activity, and the genetic restriction in this complementation implies that the antigen-binding chain has a recognition site for MHC determinant(s). The generalization can be made that the MHC-related determinants carried by the factors and the genetic restriction in their induction, in their action, and in the interchain complementation between their separated chains all map to the same region of the genome. This is intelligible on the assumption that the T-cell receptor on the cell that produces the factor has a recognition site for antigen and MHC determinants and that the antigen-binding chain of secreted factor has the same properties.

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CONTENTS

Ann. Rev. Immunol. 1986. 4 : 69–95

THE HUMAN INTERLEUKIN-2 RECEPTOR[1]

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OVERVIEW OF INTERLEUKIN-2 (IL-2) AND IL-2 RECEPTORS

In 1976, Morgan et al first reported the presence of a T-cell growth-promoting activity in the supernatants of activated T-cell cultures; they denoted this *T-cell growth factor* (TCGF) (1). In subsequent years, TCGF was characterized as a distinct biochemical entity and found to be a 15,500-dalton glycoprotein (2). TCGF was purified to homogeneity (3), molecularly cloned (4), and cDNAs expressed in eukaryotic cells. The entire gene has now been sequenced (5, 6) and localized to human chromosome 4 (7).

TCGF, or interleukin-2 (IL-2) as it is now denoted, has played a key role in the study of T-cell biology. This lymphokine has permitted both the long-term growth and the cloning of human and murine T-cell lines displaying helper and cytotoxic T-cell function (8). Furthermore, possible therapeutic effects of IL-2 in various malignancies and immunodeficiency states are now under study.

IL-2 is not produced by resting T cells; however, following activation with antigen or mitogen in the presence of the monokine interleukin-1, IL-2 is rapidly synthesized and secreted (Figure 1) (9). Following activation,

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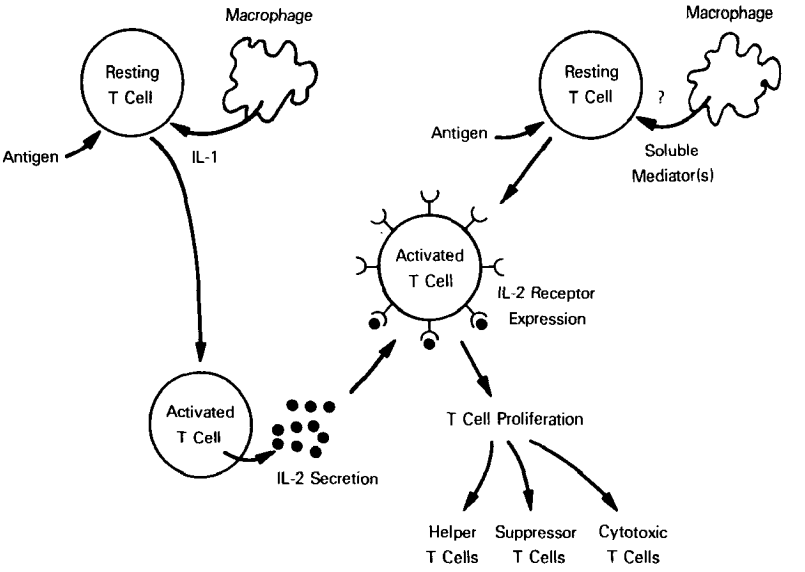

Figure 1 A model of T-cell activation and growth.

another, possibly overlapping, subpopulation of T cells expresses specific high affinity receptors for IL-2, as directly demonstrated by binding studies using radiolabeled IL-2 (10). In the presence of IL-2, these cells with receptors proliferate, resulting in the expansion of T-cell populations capable of mediating helper, suppressor, and cytotoxic functions (11, 12). Thus, the specificity of the T-cell immune response is determined by the antigen. However, after IL-2 receptors are induced, T cells proliferate in response to IL-2 in an antigen-independent manner.

IDENTIFICATION OF ANTI-IL-2-RECEPTOR MONOCLONAL ANTIBODIES

The biochemical characterization of IL-2 receptors was greatly facilitated by the identification of anti-IL-2-receptor monoclonal antibodies. The first of these antibodies was anti-Tac, prepared by Uchiyama et al by immunizing mice with intact activated T cells from a patient with a cutaneous T-cell malignancy (13). This antibody was initially determined to recognize an "activation antigen" (hence the name anti-Tac for "T-activated"). Subsequent studies indicated that the Tac antigen was the human IL-2 receptor. Evidence supporting this conclusion includes the following findings: (*a*) Anti-Tac blocked IL-2-dependent proliferation of continuous T-cell lines (14); (*b*) anti-Tac blocked the binding of radio-

labeled IL-2 to activated T cells (14); (*c*) IL-2 blocked the binding of radiolabeled anti-Tac to activated T cells (15); (*d*) IL-2 coupled to affigel and anti-Tac coupled to sepharose bound the identical 55,000-dalton glycoproteins from extracts of activated T cells (see Figure 2) (16); (*e*) both anti-Tac and anti-IL-2 antibodies precipitated the same 65,000–70,000-dalton band after IL-2 was covalently cross-linked to its receptor (15).

Anti-Tac was used to characterize the IL-2 receptor as a 50,000-dalton glycoprotein on HUT-102B2 cells and as a 55,000-dalton glycoprotein on normal activated T cells, as evaluated by migration on SDS polyacrylamide gels electrophoresed under reducing conditions (14, 15, 17, 18). We discuss in detail below the difference in apparent M_r in these cell types that is the result of difference in posttranslational processing, rather than of differences in amino acid sequence.

Other monoclonal antibodies that recognize the human IL-2 receptor have been identified. For example, B49.9 (19) and 2A3 (20) have properties very similar to anti-Tac. Monoclonal 7G7B6 (21) identifies a distinct epitope on the human IL-2 receptor. Both anti-Tac and 7G7B6 precipitate the identical 55,000-dalton protein from activated T cells and can bind simultaneously to the IL-2 receptor. Unlike anti-Tac, 7G7B6 does not inhibit IL-2-induced proliferation of T cells nor IL-2 binding to its receptor.

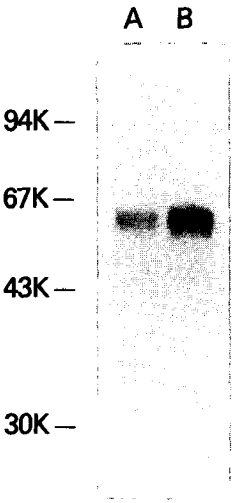

Figure 2 The IL-2 receptor has an apparent M_r of 55,000. IL-2 was covalently coupled to affigel and anti-Tac coupled to sepharose. Cellular extracts from normal activated T cells biosynthetically labeled with ^{35}S-methionine were passed over each column, the columns washed extensively, and the bound protein eluted with 2.5% acetic acid from the IL-2-affigel column (lane A) or the anti-Tac sepharose column (lane B). (Ref. 16. Reproduced with permission from the editors of the *Journal of Experimental Medicine*.)

Antimouse and -rat IL-2-receptor antibodies have also been prepared. Monoclonals 3C7 and 7D4 (22) recognize distinct epitopes on the murine receptor; 3C7 inhibits both IL-2 binding and action. In contrast, 7D4 inhibits IL-2-induced proliferation, but not IL-2 binding. ART18 recognizes the rat IL-2 receptor (23). The anti-mouse and -rat IL-2-receptor antibodies all precipitate a glycoprotein of approximately 55,000 daltons. In addition, some of the anti-IL-2-receptor antibodies coprecipitate other proteins: one between 110,000 and 120,000 daltons, and a second of approximately 180,000 daltons. It has been hypothesized that one or both of these proteins might be a subunit of a receptor complex.

PURIFICATION OF RECEPTOR PROTEIN AND MOLECULAR CLONING OF IL-2-RECEPTOR cDNAS

Three groups have employed similar protein purification and cDNA cloning strategies for the human IL-2 receptor (24–26). We present the details of our own studies. Anti-Tac was coupled to cyanogen bromide–activated sepharose and used as an affinity column to purify microgram quantities of IL-2 receptor from HUT-102B2 cells. In brief, for each protein preparation, 4×10^9 HUT-102B2 cells were extracted in NP40 containing buffer, and the cellular extracts passed first over a control antibody column and then over the anti-Tac column. The column was washed extensively with buffers of varying ionic strength, and then the bound protein was eluted with 2.5% acetic acid. This relatively simple purification scheme yielded material that was over 90% homogeneous as evaluated by densitometry scanning of silver-stained SDS-polyacrylamide gels (24). The sequence of the first 29 N-terminal amino acids of the IL-2 receptor was defined using this material, with certain positions determined or confirmed by sequencing of biosynthetically radiolabeled receptor protein. The sequence obtained was NH_2-glu-leu-cys-asp-asp-asp-pro-pro-glu-ile-pro-his-ala-thr-phe-lys-ala-met-ala-tyr-lys-glu-gly-thr-met-leu-asn-cys-glu.

Based on the amino acid sequence of residues 3–8 (underlined above), an oligonucleotide probe of length 17 and 64-fold degeneracy was synthesized. The probe was end-labeled with polynucleotide kinase and ^{32}P-ATP and used to screen 200,000 phage from a HUT-102B2 cDNA library prepared in lambda gt10. After tertiary screening, 11 candidate clones were identified, of which three were chosen for further evaluation. First, these cDNAs were used in selective hybridization experiments. The cDNAs were baked onto nitrocellulose and hybridized to total cellular HUT-102B2 cell mRNA. The selectively hybridized mRNA was eluted in water and translated in a wheat germ lysate translation system. The IL-2-receptor primary translation product was then immunoprecipitated using an anti-IL-2-receptor hetero-

antiserum prepared by immunization of rabbits with affinity-purified IL-2-receptor protein. These data confirmed that all three cDNAs were clones associated with the IL-2 receptor.

The two longest clones, denoted pIL2R3 and pIL2R4 (24), were then sequenced by the dideoxy chain termination method. The clones shared the same 5′ end, and each contained a long open reading frame, including 87 bases precisely corresponding to the 29 amino acids determined by protein sequencing. However, clone 4 differed from clone 3 in two ways. First, it was shorter (1589 bases versus 2335 bases), extending less far 3′. Second, it lacked a 216-base-pair segment within the protein coding region that was present in clone 3. Shown in Figure 3 is the amino acid sequence we deduced for the insert of pIL2R3. The 72 amino acids not encoded by clone 4 are identified in the shaded box.

In order to determine which of these clones encoded the IL-2 receptor, we

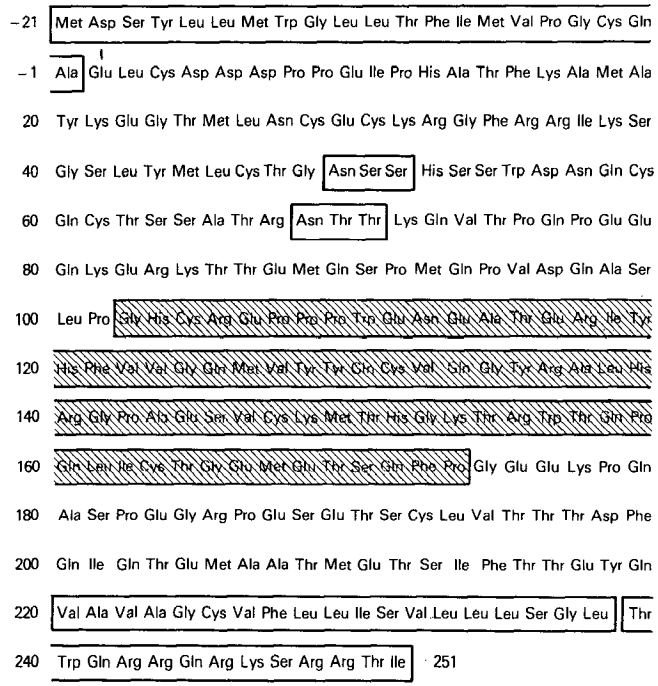

Figure 3 Deduced amino acid sequence of the IL-2 receptor based on the sequence of the HUT-102B2 cDNA (clone 3). The signal peptide (amino acids −21 to −1), two potential N-linked carbohydrate addition sites (amino acids 49 to 51 and 68 to 70), transmembrane domain (amino acids 220 to 238), and cytoplasmic domain (amino acid 239 to 251) are identified by open boxes. The deduced sequence of clone 4 lacks the 72 amino acids identified in the shaded box.

subcloned the cDNA inserts of both clones in the correct orientation into an expression vector, pcEXV-1 (provided by J. Miller and R. Germain, NIH) which contained SV40 enhancer and promoter elements. The plasmids were then transfected into COS-1 cells by calcium phosphate precipitation. At 48 hr after transfection, binding studies using both radiolabeled anti-Tac and radiolabeled IL-2 were performed to detect IL-2 receptor expression. Only clone 3 was identified to encode a functional IL-2 receptor, and a typical 50,000-dalton protein was precipitated from the surface of clone 3–transfected cells. Significantly, although the clone 4–transfected cells were transcriptionally active, no synthesized protein was detected either by binding with radiolabeled IL-2 or radiolabeled anti-Tac, or by precipitations performed with anti-Tac or the anti-IL-2-receptor heteroantiserum. Thus, it is possible either that the clone-4 mRNA was not translated efficiently or that the resultant protein was either rapidly degraded or lacked domains recognizable by IL-2, anti-Tac, or the anti-IL-2-receptor heteroantiserum. As we indicate below, clone 4 is not simply a cloning artifact; rather, it corresponds to an alternatively spliced mRNA present in both normal activated T cells and in HTLV-I-infected leukemic cells.

In Figure 3, the IL-2 receptor is demonstrated to consist of a 21-amino-acid-long signal peptide and a mature protein of 251 amino acids. The amino terminus is extracytoplasmic and the carboxy terminus intracytoplasmic. The receptor has a short (13-amino-acid-long) cytoplasmic domain that is positively charged and presumably serves an anchoring function. The protein contains a hydrophobic 19-residue membrane spanning region, as predicted by Kyte-Doolittle hydrophobicity plots, and the majority of the protein (219 amino acids) is extracytoplasmic. There are two N-linked carbohydrate addition sites and multiple potential O-linked carbohydrate addition sites. The region from amino acids 1–64 is 22% homologous to amino acids 102–174, suggesting that a historical internal gene duplication event occurred.

cDNAs encoding the murine IL-2 receptor have also been isolated by low stringency hybridization studies using human IL-2-receptor cDNAs (27, 28). The mouse IL-2-receptor sequence shares 72% DNA and 61% amino acid homology with the human IL-2 receptor. The mouse sequence also contains the internally duplicated region, indicating that this event occurred at least 50 million years ago.

Computer homology searches of the National Biomedical Research Foundation and GenBank databases revealed that the IL-2 receptor does not have significant homology to any known oncogenes. However, the IL-2 receptor shares significant amino acid homology with the recognition (binding) domain (Ba fragment) of complement factor B, as deter-

mined using a program written by Dr. Minoru Kanehisa, National Cancer Institute, that converted all GenBank DNA sequences to protein sequences. In this regard, the low density lipoprotein receptor, which shares homology with coagulation factor IX, and the IL-2 receptor both share sequence similarities to proteins that participate in a proteolytic cascade.

GENOMIC STRUCTURE

Genomic Southern blots probed with IL-2-receptor cDNAs yielded simple hybridization patterns; these made it likely that only a single gene encoded the IL-2 receptor. This was confirmed by a combination of DNA spot blot analysis and in situ chromosomal hybridization that localized the gene to chromosome 10, bands p14 → 15 (29).

Genomic phage clones spanning the human IL-2-receptor gene have been isolated, and all exons and exon-intron splice junctions sequenced (30). The gene is composed of 8 exons and 7 introns, and it spans at least 25 kilobases (see Figure 4). Much of the length of the gene is contained in intron 1, which appears to exceed 15 kilobases.

The first exon contains the 5′ untranslated region and the DNA sequence corresponding to the signal peptide. The second exon begins with amino acid number 1 of the mature protein. Exon 2 contains potential O-linked carbohydrate addition sites, and exons 2 and 3 each contain one N-linked carbohydrate addition site. Exon 2 shares significant homology with exon 4 corresponding in this way to the historical gene duplication event referred to above. Exon 4 encodes the segment that is missing in the cDNA (clone 4) derived from an alternately spliced mRNA. Thus, this alternate splicing event results in the deletion of exon 4 and the joining of exon 3 to exon 5. Exon 6 encodes the domain immediately exterior to the cell membrane, as well as additional potential O-linked carbohydrate addition

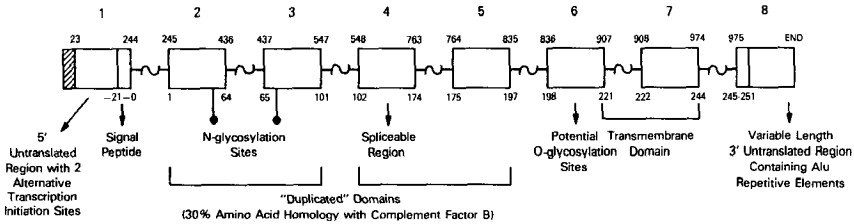

Figure 4 Genomic organization of the human IL-2-receptor gene. The locations of the transcription initiation sites, signal peptide, N-glycosylation sites, potential O-glycosylation sites, and transmembrane domain are indicated. The historically duplicated domains (exons 2 and 4) are indicated.

sites. Exon 7 encodes the transmembrane domain, and exons 7 and 8 encode the cytoplasmic domain. Exon 8 also includes the 3′ untranslated region with at least 3 polyadenylation signal sites and repetitive alu sequences between the second and third polyadenylation signal sites.

In order to identify the 5′ end of the IL-2-receptor mRNA Leonard et al conducted primer extension experiments (30). In these studies, a synthetic oligonucleotide probe corresponding to the sequence near the 5′ end of the cDNA was synthesized, end-labeled with polynucleotide kinase and ^{32}P-ATP, annealed to mRNA, and extended with reverse transcriptase. Gel analysis of the products indicated that the IL-2-receptor gene contained two principal transcription initiation sites separated by 58 bases.

To confirm that these two bands represented true transcription initiation sites, rather than areas of secondary structure in the mRNA that resulted in artificial stop sites during reverse transcription, Leonard et al carried out S1 nuclease protection assays. These experiments used a genomic DNA fragment that included the 150 most 5′ bases of the cDNA, and extended approximately 500 bases 5′ to the end of the cDNA; the fragment was subcloned into M13 bacteriophage. A radiolabeled, single-stranded probe complementary to mRNA was synthesized using the Klenow fragment of DNA polymerase I. This probe was hybridized to mRNA from IL-2-receptor positive and negative B- and T-cell lines, digested with S1 nuclease, and the resulting fragments electrophoresed on a denaturing urea/polyacrylamide gel. These studies revealed bands that corresponded to the major transcription initiation sites identified in the primer extension studies and thereby confirmed that two distinct transcription initiation sites, and thus two promoters, are used. Based on the relative intensities of the bands, no apparent preference in transcription initiation site utilization was detected.

MULTIPLE IL-2-RECEPTOR mRNA SPECIES

Although the IL-2 receptor is encoded by a single gene, Northern blots performed with IL-2-receptor cDNAs demonstrated two major size classes of mRNA with apparent sizes of 1500 and 3500 bases (24–26). To clarify whether each of these mRNAs encoded IL-2-receptor protein, mRNA was size-fractionated on a methylmercury agarose gel, the gel sliced, mRNAs from all regions of the gel were translated, and the translation products immunoprecipitated. These studies proved that mRNA from both 1500- and 3500-base–size ranges encoded the IL-2-receptor primary translation product.

It was therefore possible that the difference in size might be explained on the basis of the use of different polyadenylation signals. Careful analysis of

the sequence of the longest cDNA, pIL2R3, revealed that it contained two polyadenylation signal sequences in the 3′ untranslated region, neither of which was utilized in that particular cDNA. To investigate if the 3500-base mRNAs corresponded to the use of a further 3′ polyadenylation signal, Northern blots were hybridized with a radiolabeled probe from the 3′ end of the cDNA distal to the polyadenylation site. With this probe, only the 3500-base species was identified; this confirmed the hypothesis that the 1500-base mRNAs were produced by utilization of more 5′ polyadenylation signals (24).

S1 nuclease protection assays performed with DNA probes corresponding to a segment overlapping both proximal polyadenylation signals contained within pIL2R3 confirmed that both of these signals were also utilized in the formation of mature IL-2-receptor mRNAs. Thus, at least three different polyadenylation sites were used (see Figure 5) (31, 32).

In addition to the variable polyadenylation, however, each of the mRNAs classes presumably can exhibit alternate mRNA splicing of the 216-base region discussed above. Evidence for this was provided by S1

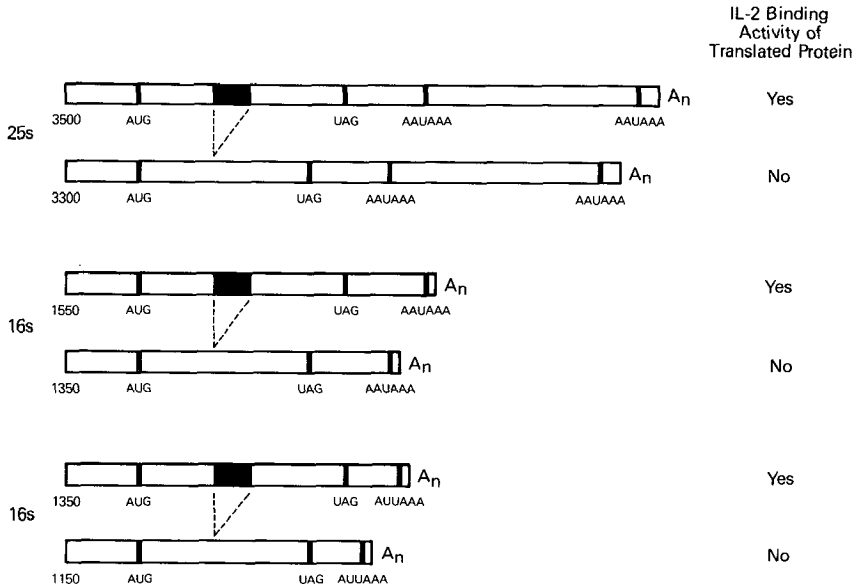

Figure 5 Six alternative transcripts of the IL-2 receptor gene. At least six different IL-2-receptor mRNAs exist. These different mRNAs are generated by the use of three different polyadenylation signals (AAUAAA or AUUAAA sequences). In addition, each mRNA may be either normally spliced or alternately spliced to omit the 216-base segment identified in black, within the protein coding region.

nuclease protection assays and by hybridizing Northern blots with radiolabeled DNA probes generated from this segment.

Thus, it appears that the 3500-base mRNA consists of at least two species, generated by normal or alternate mRNA splicing, and the 1500-base mRNAs represent at least four species: Either of two proximal polyadenylation signal sequences may be used as well as the mRNA that is either normally or alternatively spliced. Figure 5 depicts schematically these six mRNAs (see 23, 31, 32). In view of the existence of more than one transcription initiation site, however, the number of IL-2-receptor mRNAs is presumably even greater.

IL-2-RECEPTOR GENE EXPRESSION

With so many mRNAs, we sought to investigate whether any of these were preferentially synthesized under certain conditions (31, 32). Interestingly, when peripheral-blood T cells were activated with optimal concentrations of either phytohemagglutinin (PHA) or phorbol myristate acetate (PMA), the 3500-base class of mRNA was preferentially synthesized. However, when PHA and PMA were used together, both 3500- and 1500-base classes of mRNA were synthesized in approximately equal amounts. These data suggest the possibility that PHA and PMA stimulate IL-2-receptor expression through complementary mechanisms. Like lymphoblasts activated by PHA plus PMA, ATL cells infected by HTLV-I also synthesize approximately equal quantities of mRNAs of both classes and thus correspond to maximally activated normal T cells.

As noted above (see Figure 1), resting T cells do not express IL-2 receptors, but they are rapidly induced following activation with PHA. Nuclear run-off experiments were used to evaluate whether this induction was regulated at the level of transcription (32). In nuclei isolated from resting cells, no IL-2-receptor mRNA was detected; however, within 3 hr of activation (the earliest time studied), IL-2-receptor gene transcription was occurring at a high level. Transcription continued at a high level for 24 hr and then declined. In contrast, when peripheral-blood T cells were activated with PHA, IL-2-receptor mRNA expression reached a maximum at between 8 and 24 hr and then declined. Analysis of the data indicated that an increase in transcription could account for the initial increase in IL-2-receptor mRNA levels; however, these appeared to fall before there was a substantial decline in IL-2-receptor nuclear transcription. Thus, it is possible that post-transcriptional rates of mRNA processing or degradation are also important in regulation of IL-2-receptor gene expression.

Although IL-2-receptor mRNA levels were maximal between 8 and 24 hr, receptor expression at the cell surface did not peak until 48–72 hours.

The long lag between the peak in mRNA levels and protein levels is somewhat surprising and presently unexplained.

SEQUENTIAL GENE EXPRESSION DURING T-LYMPHOCYTE ACTIVATION

Using nuclear transcription analysis, Krönke and colleagues (33) have studied the temporal sequence of gene activation following stimulation of human T cells with PHA and PMA (Figure 6). These authors have found that the proto-oncogene, c-myc, was activated rapidly and reached peak transcription within 6–9 hr, results consistent with those of earlier studies of Kelly et al (34). c-myc expression was followed sequentially by transcription of the genes encoding the IL-2 receptor and interferon-gamma that peaked at 9–15 hours after mitogen addition. IL-2 gene transcription occurred later, reaching optimal levels at approximately 24 hr. Thus, activation of the IL-2-receptor gene temporally preceded IL-2 gene expression. Transcription of each of these four genes was not inhibited by the addition of cycloheximide in sufficient quantities to block >98% of protein synthesis. These data indicated that the protein products of these genes were not required for the subsequent activation of transcription of the other genes; for example, the c-myc protein was not necessary for transcription of the IL-2 gene. In contrast to this family of genes, transcribed early and independently of protein synthesis, the transferrin receptor gene was not expressed until 24 hr after mitogen activation and did not reach peak levels until 48 hr. Addition of cycloheximide at the initiation of culture completely blocked transferrin receptor gene transcription; this indicated a dependence on protein synthesis. These results were in agreement with those of the earlier studies of Neckers & Cossman, who reported that transferrin

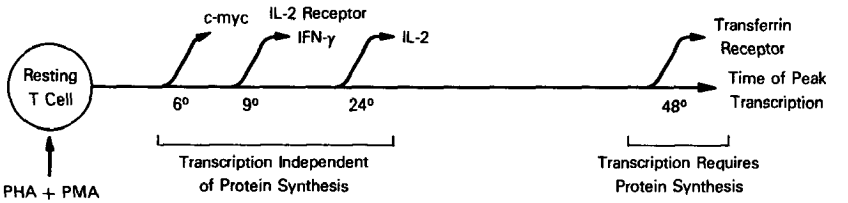

Figure 6 Sequential gene expression in activated human T cells. Nuclear runoff assays were performed with isolated nuclei obtained from human T cells incubated for varying periods of time with PHA and PMA. The level of transcription of c-myc, IL-2 receptor, interferon-gamma, IL-2, and transferrin receptor genes was measured at times from 0–72 hr after activation. Time of peak transcription for each of these genes is schematically shown. Identical studies were performed in the presence of cycloheximide to assess the dependence of gene transcription upon intact protein synthesis.

receptor expression required a prior interaction of IL-2 with the IL-2 receptor (35).

INDUCTION OF IL-2-RECEPTOR REEXPRESSION

As noted earlier, mitogen-induced, IL-2-receptor expression is characterized by an early rise followed by a later decline in cell surface receptor number that is paralleled by changes in the proliferative capacity of these cells. After 10–12 days of culture, IL-2-receptor number is only 10–20% of the peak level present on activated T lymphocytes at day 2–3 (36, 37). These senescent T cells, however, remain susceptible to signals that result in the reexpression of IL-2 receptors and restoration of cellular proliferation (36–40). Three general types of activation signals appear to stimulate IL-2-receptor reexpression (Figure 7, left panel). First, the greatest increase in receptor number was obtained by reexposure of the cells to mitogenic lectin (37, 41). For example, PHA restimulation resulted in a 2–10-fold increase in IL-2-receptor number within 24 hr. As studies with actinomycin D and cycloheximide indicate, PHA-induced reexpression of IL-2 receptors was

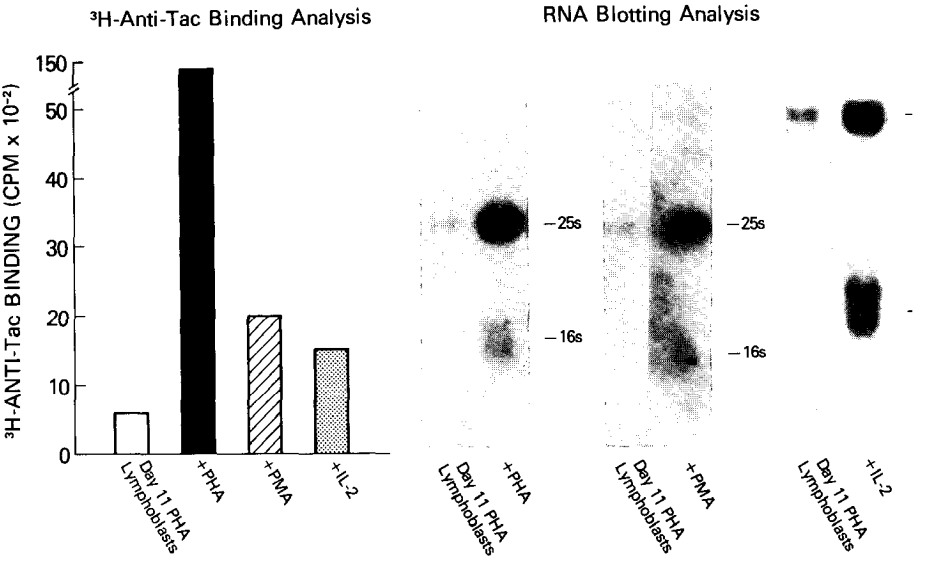

Figure 7 Induction of IL-2 receptor reexpression. Left Panel: Day 11 PHA lymphoblasts that had lost >80% of their surface-IL-2 receptors were restimulated with PHA, PMA, or IL-2, and receptor expression was measured 12–24 hr later in ^{3}H-anti-Tac-binding assays. Right Panel: Total cellular RNA was isolated from Day 11 lymphoblasts that were stimulated with either medium, PHA, PMA, or IL-2 for 12 hr at 37°C. 20 micrograms of RNA from each sample was size fractionated on formaldehyde gels and transferred to nylon membranes (Gene screen, NEN). The blots were then hybridized with radiolabeled IL-2-receptor cDNA probes.

dependent upon de novo RNA and protein synthesis, and this suggests that receptor display was not solely the result of mobilization of preformed intracellular receptors to the cell surface. Second, addition of the phorbol ester, PMA, also produced augmented IL-2-receptor display. Similarly, phospholipase C and synthetic congeners of diacylglycerol each stimulated increased IL-2-receptor expression (37, 41). These agents activate protein kinase C, suggesting that this kinase may play an important role in the regulation of IL-2-receptor expression. Third, addition of IL-2 also promoted augmented IL-2-receptor display in both human and murine T cells (42–44). Other investigators have also reported that IL-2 amplifies IL-2-receptor expression in freshly isolated, resting lymphocytes activated under suboptimal conditions (42, 45, 46). Similarly, IL-2-induced increases in IL-2-receptor number have been detected in a cloned B-cell line (5B4) that constitutively expresses small numbers of IL-2 receptors (47). These effects of IL-2 occurred at ligand concentrations that preferentially result in binding to the high, but not low, affinity class of IL-2 receptors (43, 47; see below for discussion of high and low affinity receptors). Low affinity receptors are preferentially induced in these reexpression experiments. We discuss later the possibility that this difference relates in part to the finding that the high, but not low, affinity IL-2 receptors undergo receptor-mediated endocytosis (48).

In Northern blotting studies (Figure 7, right panel), PHA, PMA, and IL-2 each augmented the expression of IL-2-receptor mRNA. Measured in nuclear runoff assays, IL-2-receptor gene transcription also increased. IL-2-mediated reexpression of receptors was also associated with increased levels of c-myc and transferrin receptor mRNA but did not alter the levels of mRNA encoding the beta chain of the T-cell-antigen receptor (43). Within 48–72 hr after activation with PHA, PMA, or IL-2, the IL-2-receptor numbers again declined, which indicates that both the initial induction and subsequent reexpression of IL-2 receptors is a transient, nonsustained event. Despite the capacity of IL-2 to mediate upregulation of IL-2-receptor number in these senescent T cells, this ligand did not prevent the initial and physiologic decline in receptor levels. This finding may reflect active but transient repression of IL-2-receptor gene transcription that results in the initial decline in receptor number (43). Similar repressor activity has been implicated in the transient expression of the IL-2 gene in activated T cells (49).

POSTTRANSLATIONAL PROCESSING OF HUMAN IL-2-RECEPTOR PROTEIN

Attempts to immunoprecipitate the primary translation product of the human IL-2 receptor with currently available monoclonal antireceptor

antibodies, used either alone or in combination, have proven unsuccessful (W. C. Greene, W. J. Leonard, unpublished observations). However, following immunization of a rabbit with small amounts of receptor protein purified by anti-Tac immunoaffinity chromatography, a polyclonal antiserum was produced that reacted with the primary translation product generated in a wheat germ lysate system; this process used mRNA from both normal activated T cells and HTLV-I–infected HUT 102B2 cells (24). SDS-PAGE analysis indicated that the primary translation product had an apparent M_r of 34,500 in both normal and leukemic activated T cells (Figure 8). After pulse-chase labeling with ^{35}S-methioninine in the presence of tunicamycin, which blocks N-linked glycosylation, a 33,000 M_r protein (p33) was identified with anti-Tac. This protein corresponded to the unmodified IL-2-receptor peptide (15, 18). Thus, signal peptidase cleavage of the 21 amino acid hydrophobic leader sequence and perhaps other post-translational folding events are required for recognition of the protein by anti-Tac. In the absence of tunicamycin, p33 was not identified; rather, two precursor proteins of M_r 35,000 and 37,000 (p35 and p37) were present. This suggests cotranslational processing of p33 by N-linked glycosylation (15, 18; Figure 8). That relationship was confirmed in studies with endoglycosidase F, which cleaves N-linked sugar moieties, by conversion of p35/p37 to p33. Analysis of the primary amino acid sequence of the IL-2 receptor that had been deduced indicates the presence of two N-linked glycosylation sites (24). Pulse-chase studies also demonstrated that the N-linked precursors were subsequently exported to the Golgi apparatus where O-linked sugar and sialic acid were added; that resulted in a saltatory increase in apparent receptor size to an M_r of 55,000. These Golgi-associated processing events were blocked by addition of the carboxylic

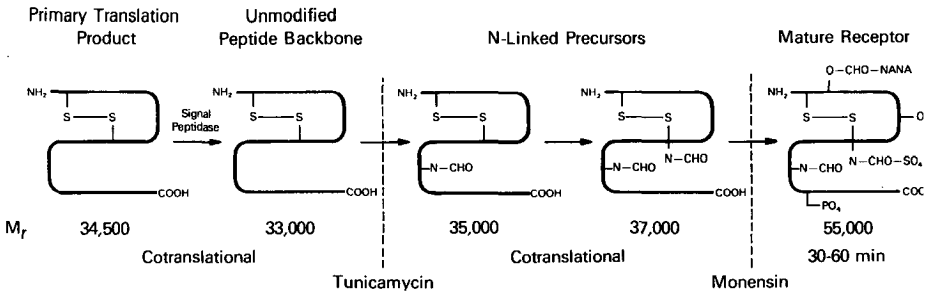

Figure 8 A schematic depicting the pattern and time course of posttranslational processing of the human IL-2 receptor as determined by pulse-chase labeling studies performed in the presence and absence of tunicamycin and monensin, digestion with endoglycosidase F and neuraminidase, and labeling with ^{35}S-sulfuric acid and ^{32}P-orthophosphoric acid. The primary translation product was immunoprecipitated with an anti-IL-2-receptor heteroantiserum, while other forms were immunoprecipitated with anti-Tac or immobilized IL-2.

ionophore, monensin; the result was an accumulation of p37, but not p35 nor p33 (18). These data suggest that p35 is normally processed to p37.

In addition to the introduction of N- and O-linked carbohydrate, the IL-2-receptor protein is posttranslationally modified by sulfation (18). At present, it is unknown whether the sulfate is introduced into carbohydrate (usually N-linked sugar) or protein (occasionally tyrosine residues). However, after ^{35}S-sulfuric acid labeling and hydrolysis of the labeled protein, essentially all of the radioactivity was precipitable with barium chloride; this indicates that the label was incorporated as sulfate rather than sulfide. The difference in apparent size of the HUT 102B2 compared to normal activated T-cell IL-2 receptor (50,000 M_r versus 55,000 M_r) is at least in part related to diminished addition of sulfate as well as sialic acid to the leukemic receptor protein. The biochemical basis for this altered posttranslational processing is not completely understood. Comparison of the amino acid sequence deduced for the HUT 102B2 receptor (cDNA sequence) and normal receptor (genomic sequence) has revealed no apparent differences (30). Thus, the presence of a point mutation resulting in the removal of a site for sulfation or glycosylation does not appear to be likely. Furthermore, other glycoproteins such as T4 and T9 are normally processed in HUT 102B2 cells indicating that these leukemic cells do not display global defects in posttranslational processing of membrane proteins.

Following these extensive posttranslational processing events, the mature receptor protein (M_r 55,000 in normal activated T cells) is displayed on the cell surface. Constitutive phosphorylation of this mature receptor protein has also been detected (18, 50). The primary site of phosphorylation involves the serine residue at position 247 located within the intracytoplasmic domain. Thus far, evidence for IL-2-induced phosphorylation of the IL-2 receptor has not been obtained but cannot yet be excluded. Certainly, the intracytoplasmic domain of the p55 IL-2-binding protein is insufficient in length to encode a kinase activity; this makes IL-2-receptor-induced autophosphorylation unlikely.

Both IL-2 and anti-Tac immobilized on bead supports react with p33-, p35-, and p37-precursor forms of the receptor, but neither reacts with the primary translation product (18). Therefore, the extensive posttranslational processing of this receptor is not obligately required for IL-2 binding; however, it is possible that these processing events alter the resultant affinity of the receptor for its ligand.

IL-2 RECEPTORS ON B CELLS AND THYMOCYTES

Although it was initially assumed that IL-2 receptors were uniquely expressed on T lymphocytes, various lines of evidence began to suggest that

IL-2 receptors were present on at least some B cells. Low-level binding of ^{125}I-anti-Tac was detected on the Burkitt lymphoma cell line Concepcion (unpublished observations) as early as 1982, and Ortega et al (22) noted that IL-2 receptors were present on lipopolysaccharide-stimulated B lymphoblasts. As a B-cell malignancy (51) determined by rearrangement of both heavy and light chain immunoglobulin genes, hairy cell leukemia expressed Tac antigen. These studies have been extended now to demonstrate that IL-2 may induce both proliferation (52) and differentiation of B cells (53). Waldmann et al (47) demonstrated high-affinity IL-2-receptor sites on a cloned, activated B-cell line. Further, they provided evidence for upregulation of IL-2 receptors on that cell line in response to exogenous IL-2. The IL-2 receptors on T cells and on B cells are identical when evaluated by chymotryptic peptide maps (54). The in vivo role of IL-2 in B-cell differentiation and proliferation remains unknown, but the concensus now is that IL-2 receptors may be expressed on B cells and that at least in vitro, B cells can be responsive to purified IL-2.

IL-2 receptors have also been detected on thymocytes (55, 56). Ceredig et al (55) have described IL-2 receptors on 50% of the Lyt2 negative, L3T4 negative thymic "stem cells." These cells are distributed throughout the thymic cortex and medulla. Raulet (56) has identified IL-2 receptors on 30% of these same cells and, in addition, on 50% of immature fetal thymocytes. These cells proliferate weakly in response to IL-2 or concanavalin A, but strongly when costimulated with both reagents. It is unknown what, if any, in vivo role IL-2 receptors have with thymocytes, but it is possible that they may be involved in the positive selection of thymocytes during thymic education.

HIGH AND LOW AFFINITY RECEPTORS FOR IL-2

The initial radiolabeled, IL-2-binding studies of Robb et al indicated that normal activated human T cells displayed 2000–4000 IL-2 receptors per cell (10). IL-2 interacted with these receptors with unexpectedly high affinity (apparent K_d of 2–5 pM). In contrast to these results, binding studies performed with radiolabeled anti-Tac demonstrated 30,000–60,000 receptors per cell (37, 57). Furthermore, while virtually all of the anti-Tac binding could be blocked with IL-2, the quantities of IL-2 required were much greater than predicted by the apparent K_d of IL-2 binding (15, 58). This discrepancy in receptor number measured in the two assays was determined not to be the result of F_c receptor binding of antibody or of partial occupancy of IL-2 receptors by endogenously produced IL-2; neither was it secondary to significant errors in the estimation of the specific activity of either ligand. Therefore, the possible existence of two affinity classes of IL-2

receptors was considered. To address this possibility, binding studies were repeated with additions of much greater amounts of IL-2 (59). These studies revealed a second, more numerous class of IL-2 receptors that bound IL-2 with a lower apparent affinity (K_d of 5–20 nM). In general, the high affinity receptors comprised only 5–10% of the total number of IL-2 receptors, while 90–95% of the receptors were of low affinity. In contrast, these different affinity classes of IL-2 receptors were not discriminated in the anti-Tac binding assays. The sum of the high and low affinity IL-2 receptors measured with radiolabeled IL-2 approximated within a factor of 2 the number of receptors obtained in the anti-Tac binding studies. This level of agreement is certainly within the margin of error for estimation of specific activity of each of the radiolabeled ligands. High and low affinity receptors for other growth factors and hormones have also been recognized in other experimental systems (60–62). However, the difference in K_d of the high and low affinity IL-2 receptors was considerably greater (approximately 1000-fold) than that encountered in other systems (50–100-fold difference). Recent studies by Lowenthal and colleagues (63) have similarly detected both high and low affinity forms of the IL-2 receptor on the surface of activated T and B cells; however, these authors estimate that the apparent difference in K_d is only 75-fold for activated T cells (K_d of 17 pM versus 1.27 nM). These findings may reflect methodological differences of washing in the assays performed by the two groups (63).

Notwithstanding, the growth-promoting effects of IL-2 appear to be mediated by the interaction of ligand with the high affinity receptors (10, 59). The biological function of the low affinity IL-2 receptors, if any, remains now unresolved. As noted earlier, the high affinity, but not low affinity, IL-2 receptors undergo receptor-mediated endocytosis (48). These studies further highlight a fundamental structural and functional difference in the high and low affinity IL-2 receptors. Whether the internalization of the high affinity form of the receptor is involved in signal transduction is unknown.

The structural differences that distinguish the high and low affinity IL-2 receptors remain undefined. At least three possibilities for these different receptor affinity classes exist. First, it is possible that the high affinity IL-2 receptor is actually composed of a complex of proteins. This model is particularly attractive in terms of providing a mechanism for receptor signal transduction. As noted earlier, the intracytoplasmic domain of the 55,000-dalton, IL-2-binding protein is only 13 amino acids in length and contains no obvious enzymatic activity. In particular, tyrosine kinase activity, found with virtually all other growth-factor receptors, is not associated with this intracytoplasmic domain. Perhaps other proteins complexed with the M_r 55,000-dalton protein are required for high affinity IL-2 binding and propagation of the signal for growth into the cell. In this

regard, anti-Tac immunoprecipitation of activated T cells biosynthetically labeled with ^{35}S-methionine has revealed small quantities of two additional proteins termed p113 and p180 (14). These proteins are not labeled by cell surface iodination, nor do they appear to be glycosylated; however, each is phosphorylated. It is possible that one or both of these proteins, or other yet unrecognized proteins, together with p55 form a receptor complex capable of binding IL-2 with high affinity and transducing the growth-promoting signal.

Second, since the IL-2 receptor undergoes extensive post-translational modification, it is conceivable that the high affinity IL-2 receptor is produced by a select form of processing. For example, potential differences in phosphorylation or sulfation could alter the affinity of the receptor for its ligand. This model, however, does not provide an explanation for signal transduction through the short intracytoplasmic domain of p55.

The third possibility is that the high and low affinity receptors are encoded by different genes and that the cDNAs isolated correspond to the low affinity receptor form. In this regard, expression of the IL-2-receptor cDNA in mouse L cells results in the exclusive display of low affinity IL-2 receptors (64). However, this finding could also reflect the lack of the appropriate companion proteins required to form the high affinity, IL-2-receptor complex, or it may reflect the absence of appropriate posttranslational processing within the L cells. The two-gene hypothesis is also made less likely by the presence of shared monoclonal antibody–binding epitopes between the high and low affinity forms of the IL-2 receptor. In the human system, both high and low affinity receptors react with anti-Tac and probably 7G7B6. In the murine system, both receptors react with two different monoclonals (3C7 and 7D4) that recognize different epitopes on the mouse IL-2 receptor (22). Thus, if the high and low affinity IL-2 receptors are different proteins, they must share antigenic sites recognized by these monoclonal antibodies.

IL-2-RECEPTOR EXPRESSION IN HTLV-I-INDUCED ADULT T-CELL LEUKEMIA

Human T-Lymphotrophic Virus-I (HTLV-I) is a type C retrovirus capable of infecting and transforming mature T cells (65–67). HTLV-I has been implicated as the etiologic agent (68) in the Adult T-Cell Leukemia (ATL) (69). This aggressive and often fatal leukemia is geographically clustered in HTLV-I endemic areas including the southwestern part of Japan, the Caribbean basin, southeastern United States, and sub-Saharan Africa. Patients with this leukemia may present with epidermal or dermal leukemic T-cell infiltrates, hypercalcemia, osteolytic bone lesions, infection with

opportunistic organisms, hepatosplenomegaly, and pulmonary infiltrates (69, 70).

A unique feature of the HTLV-I-infected T-cell lines is the constitutive expression of 5–10-fold more IL-2 receptors than are present on optimally activated PHA T lymphoblasts (57, 71). Furthermore, some, but not all, ATL cell lines display IL-2 receptors that are abnormal in size, apparently due to differences in posttranslational processing of the receptor (18, 72).

The mechanisms accounting for high level IL-2-receptor display in HTLV-I-infected T cells and the potential role of these receptors in the growth of these leukemic cells remain unknown. Northern blotting studies with IL-2 cDNA probes have demonstrated that the majority of ATL cell lines do not transcribe IL-2 mRNA; thus leukemic T-cell growth based on autocrine stimulation is unlikely (73). Furthermore, the majority of established ATL cell lines are not dependent on IL-2 for cell growth, nor is their proliferation blocked by anti-Tac. However, these data do not preclude the possibility that circulating IL-2 may play an important role in leukemic T-cell growth at an earlier stage in the ontogeny of this leukemia. In this regard, a period of growth-factor dependence followed by the progressive development of growth-factor independence has been demonstrated in other tumor systems.

As noted earlier, the human IL-2-receptor gene has been localized to chromosome 10 bands p14 → 15 (29). Karyotypic analyses of several ATL cell lines, as well as freshly isolated leukemic T cells, have not revealed consistent abnormalities of chromosome 10. Thus, it appears unlikely that augmented IL-2-receptor expression is the result of chromosomal translocation involving the IL-2-receptor gene locus. Furthermore, Southern blotting analysis has provided no evidence for rearrangements or amplifications of the IL-2-receptor gene in several ATL cell lines (31). Analysis of HTLV-I integration sites in a variety of freshly isolated leukemic cells, as well as in ATL cell lines, has demonstrated that this virus does not integrate at unique sites (74). Thus, it is quite unlikely that IL-2-receptor expression reflects *cis*-activation of the IL-2-receptor gene via viral promoter insertion.

How then does HTLV-I activate IL-2-receptor expression? Studies are presently focusing on the possibility that the virus elaborates a factor(s) that, acting in a *trans*-manner, might activate transcription of the IL-2-receptor gene (Figure 9). In this regard, Seiki et al have completely sequenced one HTLV-I viral isolate and found evidence for four open reading frames in a region termed pX located near the 3′ end of the virus (75). Several groups (76–80) have studied the protein products of this pX region. One of these genes is referred to as LOR (long open reading frame). Utilizing plasmid constructs of the regulatory LTR region of HTLV-I linked to the *chloramphenicol-acetyl-transferase* (CAT) gene, Sodroski et al

have demonstrated that the LOR protein (M_r of 42,000 daltons in HTLV-I-infected T cells) is capable of activating transcription of genes controlled by the retroviral LTR in a *trans* manner (81, Figure 9). Studies are now focused on whether the LOR protein also *trans*-activates transcription of cellular genes, including possibly the IL-2 receptor, which leads to T-cell transformation and uncontrolled growth (Figure 9). A direct action of the LOR protein on cellular genes has not yet been proven. Yodoi and coworkers have described the production by ATL cells of a soluble mediator termed ADF (*A*dult T Cell Leukemia *D*erived *F*actor) that is capable of inducing IL-2-receptor expression in select target T-cell lines (82). It is possible that ADF is involved in the augmented expression of IL-2 receptors in this leukemia. It is not yet known whether ADF production is linked to preceding action of the LOR protein.

While the mechanism of viral induction of IL-2-receptor display remains elusive, Northern blotting studies indicate that ATL cell lines constitutively express large quantities of IL-2-receptor mRNA (31). S1 nuclease studies of posttranscriptional splicing and use of the three different polyadenylation signal sites indicate that these events occur normally in ATL cells (31). Nuclear run-off studies have demonstrated that the IL-2-receptor gene is

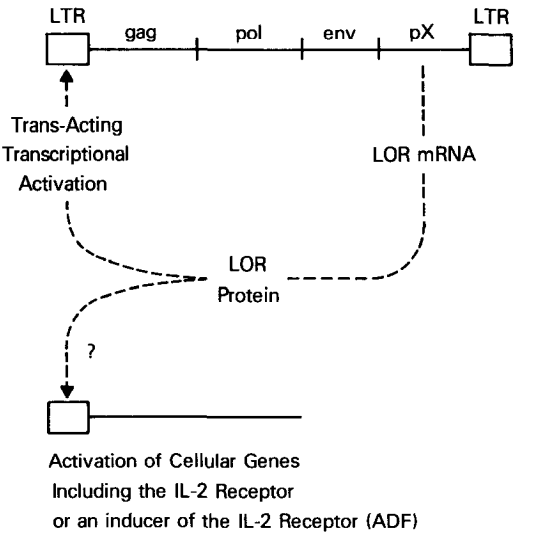

Figure 9 Hypothetical model for the mechanism of constitutive IL-2-receptor expression in HTLV-I-infected leukemic T cells.

transcribed at a high level in ATL cells in the absence of added mitogens. However, the addition of PHA and PMA, which activate IL-2-receptor expression in normal T cells, paradoxically inhibits (60–80%) IL-2-receptor gene transcription in the HTLV-I-infected leukemic T cells (31). This inhibition is not the result of generalized cytotoxic effects of these stimulants, as transcription of the transferrin receptor and c-myc genes is unaltered. Krönke et al have speculated that the inhibitory effects of mitogens in ATL cells might represent activation of a repressor mechanism that selectively inhibits IL-2-receptor gene transcription (31). This same repressor system might also mediate the physiologic decline in IL-2-receptor number occurring in normal T cells.

To further investigate the constitutive display of IL-2 receptors in ATL cells, Leonard et al analyzed IL-2 receptor promoter structure and function in these cells by using primer extension analysis and S1 nuclease protection assays (30). Primer extension studies in which a 5′ radiolabeled oligonucleotide probe was annealed to mRNA and extended with reverse transcriptase indicated the presence of the two major sites for initiation of transcription, as noted above, in both normal and ATL leukemic T cells (30). However, a third transcription initiation site was detected in ATL cells that was not apparently utilized in normal activated T cells. S1 nuclease protection assays confirmed these two principal shared transcription initiation sites and the unique site in ATL cells. Together, these data suggest that both normal and ATL leukemic T cells utilize two separate major promoters while ATL cells also utilize a third, normally cryptic, promoter. The presence of a third IL-2-receptor promoter in ATL cells is intriguing, but these studies demonstrate that this promoter is considerably less active than the two major promoters. Thus, this third promoter would not appear to provide a full explanation for the deregulated, amplified expression of IL-2 receptors encountered in this leukemia.

ANTI-IL-2-RECEPTOR ANTIBODIES AS POSSIBLE THERAPEUTIC AGENTS IN ATL

No satisfactory chemotherapy for the treatment of patients with Adult T-Cell Leukemia has been identified. The display of large numbers of IL-2 receptors by these leukemic T cells coupled with the availability of monoclonal antibodies that bind to these receptors with high affinity has made immunotherapy an attractive therapeutic approach. Waldmann and associates are presently evaluating the effects of unmodified anti-Tac antibody administered intravenously to patients with this leukemia. Preliminary results suggest that patients with the more common aggressive form of this disease are not significantly benefited by this therapy. This

finding is consistent with the inability of anti-Tac in vitro to inhibit the growth of IL-2-independent ATL cell lines. However, a subgroup of these patients may present with a more smoldering, less aggressive form of leukemia. One such patient has responded to parenteral anti-Tac therapy with marked regression of skin lesions and a decline in the number of Tac-positive leukemic T cells circulating in the peripheral blood (T. A. Waldmann, unpublished observations).

In view of the apparent lack of therapeutic effects with unmodified anti-Tac in ATL patients with aggressive disease, in vitro and in vivo studies have been initiated that employ anti-Tac covalently coupled to select toxins. Krönke et al have demonstrated that anti-Tac-ricin A-chain immunotoxins selectively kill HTLV-I-infected leukemic T cells in vitro (83). Furthermore, immunotoxin killing was enhanced in the presence of lysosomotropic agents such as ammonium chloride, chloroquine, and monensin. In regard to the possible use of such immunotoxins to "purge" leukemic bone marrow, Krönke et al have observed, in clonogenic assays, that doses of anti-Tac ricin A, which kill greater than 99.9% of the ATL cells, had little or no inhibitory effect on the growth of bone marrow progenitor cells (83, and M. Krönke, W. C. Greene, unpublished observations).

Fitzgerald et al have found similar cytotoxic effects of anti-Tac covalently coupled to pseudomonas exotoxin (84). Therapeutic trials of anti-Tac-pseudomonas exotoxin conjugates in the treatment of patients with ATL are presently underway at the National Cancer Institute.

IMMUNOSUPPRESSIVE EFFECTS OF ANTI-IL-2-RECEPTOR ANTIBODIES

Depper et al have demonstrated that anti-Tac inhibits most of the proliferative responses of human T cells stimulated with soluble, autologous, or allogeneic antigens (85). Consistent with a functional blockade of the IL-2 receptor, this inhibition of proliferation was reversed by the addition of excess amounts of purified IL-2. Similar results have been obtained by Malek et al using monoclonal anti-IL-2-receptor antibodies in the murine system (86). Anti-Tac also inhibited T-cell proliferation induced by the mitogenic lectins—concanavalin A, pokeweed mitogen, and to a lesser extent, phytohemagglutinin. In addition, anti-Tac abrogated the IL-2-dependent maturation of cytotoxic T-cell precursors into cytotoxic effector T cells (85). In terms of effects on humoral immunity, anti-Tac inhibited the production of B-cell immunoglobulin activated by pokeweed mitogen. As the pokeweed mitogen–induced response is dependent upon helper T cells, the site of anti-Tac action in this inhibition is unclear. The monoclonal

antibody could be reacting either with the required helper T cells or, alternatively, with the activated B cells now known to display IL-2 receptors.

The in vitro inhibition of the development of cytotoxic T lymphocytes has recently been explored in vivo in a rodent model of allograft transplantation by Kirkman and colleagues (87). These investigators have found that anti-IL-2-receptor antibodies markedly diminish rejection of transplanted allogeneic hearts in this animal model. These data suggest the possibility that anti-Tac or similar monoclonal antibodies might be of therapeutic utility in the prevention of allograft rejection in humans.

CONCLUSIONS

In this review, we have attempted to discuss salient molecular, biochemical, and functional features of the human interleukin-2 receptor. This surface receptor plays an important role in the growth of normal human T cells and perhaps B cells as well. The development of monoclonal antibodies that react specifically with this receptor has permitted biochemical characterization of both precursor and mature forms of this receptor as well as the recognition that these receptors are displayed in high and low affinity states. The purification and NH_2 terminal amino acid sequencing of the receptor protein has allowed the successful isolation of cDNAs encoding the IL-2 receptor. The availability of these molecular probes has led to the elucidation of the structure and chromosomal location of the IL-2-receptor gene as well as insights into the regulation of receptor gene expression. The recognized deregulated expression of IL-2 receptors in HTLV-I-induced Adult-T Cell Leukemia remains an intriguing but unexplained phenomenon. Studies are underway to define whether these receptors participate in the uncontrolled growth of these cells or alternatively occur as a byproduct of virus-induced transformation of these T cells. Regardless of the unresolved relationship of HTLV-I to the IL-2 receptor, the presence of these receptors on the surface of ATL cells, often in large numbers, has provided novel approaches to the therapy of this leukemia, approaches that employ unmodified anti-Tac or anti-Tac covalently complexed with toxins, drugs, or radionuclides. Certainly the availability of cloned genes and cDNAs for both IL-2 and its cellular receptor, appropriate monoclonal antibodies, and large amounts of purified IL-2, make this growth factor–receptor system particularly attractive for future study of transduction of growth-promoting signals. Similarly, as the IL-2 receptor is an activation antigen, this receptor may serve as an excellent model system for the general study of eukaryotic gene activation and expression.

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CONTENTS

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LYMPHADENOPATHY-ASSOCIATED-VIRUS INFECTION AND ACQUIRED IMMUNODEFICIENCY SYNDROME

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INTRODUCTION

Tremendous accomplishments have followed the original clinical description in 1981 of the acquired immunodeficiency syndrome (AIDS) and other apparently related syndromes (1–4). The major advance has been the isolation and characterization of a novel human retrovirus, lymphadenopathy-associated virus (LAV) (5–7). The belief that it is the causative agent of these new diseases stems from a large body of evidence from viro- and sero-epidemiological studies, and this belief is supported by the major characteristics of its biological properties.

Although a vaccine is still several years away, therapeutical breakthroughs are presently expected from the use of drugs that are active against such viruses (8–10). However, many questions remain unanswered. Besides the problem of precise taxonomic classification of LAV, these questions concern both the pathophysiological mechanisms that lead from viral infection to disease and the capacity of a given host actually to mount protective immune responses against this virus.

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THE DISEASE

Brief Description

AIDS is an epidemic form of immunodeficiency that results in high susceptibility to opportunistic infections or to such malignant diseases as Kaposi's sarcoma (11, 12). Up to now, it has been observed most frequently among individuals belonging to "at risk" populations: homosexual men, intravenous drug abusers, and blood product recipients, especially hemophiliacs (13). It has also occurred as an epidemic that strikes males, females, and infants in Central Africa and in Haiti (14–16). A variety of other chronic or acute (17) conditions have also been noted with increasing frequency in the same high-risk populations (4, 18). These conditions include nonspecific symptoms such as persistent generalized hyperplastic lymphadenopathy, recurrent fever, diarrhea, and weight loss, and they have been called *lymphadenopathy syndrome* (LAS) or *AIDS-related complex* (ARC), according to their gravity. They appear to be either prodromata or milder forms of AIDS. Although clinical presentations vary, the overall pattern of biological abnormalities found in all these syndromes is similar, albeit with different intensities (19, 20).

Major Immunologic Abnormalities

A large number of immunologic abnormalities have been described in these conditions (21, 22), but the major characteristic of AIDS is a persistent, profound, quantitative, and functional defect of the helper-inducer T-lymphocyte subset that is defined by the expression of the CD4 antigen (also termed T4) at the cell membrane (23). At least some of these defects may be related both to the decrease in the number of T-cell precursors present in the bone marrow or blood (24) and to abnormalities of their differentiation capacities (25). Altogether, deficient CD4+ lymphocyte function appears central to most of the functional defects that affect the other cells involved in the immune response (22).

A relatively constant feature of AIDS is the host's decreased capability to secrete lymphokines (22), especially Interleukin-2 (IL-2) (26) upon T-cell stimulation. Such inability may be one of the mechanisms of immune deficiency in AIDS patients. Nevertheless, although the addition of exogenous IL-2 has been shown to increase some immune function in vitro, it has as yet usually been unable to restore these responses to normal levels (26, 27). This argues against the existence of a pure selective defect in IL-2 production, all the more so since clinical trials with this molecule have thus far proven to be unsatisfactory. Moreover, a reduced capability of IL-2 receptor (T-activated antigen-Tac) expression exists among activated T lymphocytes (26, 28). Therefore, one can consider that both low IL-2

production and reduced numbers of Tac+ responding cells contribute to immune deficiency in this disease. These mechanisms are compatible with the retroviral etiology of AIDS and LAS in a manner similar to that described for animal retroviruses, which depress T-cell function by reducing both secretion of and response to IL-2 (29–31).

THE VIRUS

Soon after the disease was identified, epidemiological data strongly suggested that AIDS was caused by an infectious agent. Transmission of the disease by filtered blood products, as in hemophiliacs, for example, indicated that this agent was most likely a virus. Retroviruses were good candidates since some animal leukemogenic viruses are known to cause immune deficiency (30). The hypothesis that human T-cell leukemia viruses (HTLV-I and -II), just discovered, were involved seemed at first attractive (32, 33). However, HTLV-I was not frequently isolated from AIDS patients (34); antibodies to HTLV proteins were rarely detected in European patients (35–37); and AIDS was nonexistent in Japan although HTLV-associated adult T-cell leukemia is a recognized disease in that country.

All these negative data suggested that a new retrovirus was the cause of AIDS.

Isolation of LAV

The virus was first isolated at the beginning of 1983 from lymph node lymphocytes of a homosexual man with LAS, which were cultured under optimal conditions for the growth of T lymphocytes (5). It is why this virus was named lymphadenopathy-associated virus (LAV). Thereafter, it was obtained from patients presenting with the various clinical forms of AIDS (opportunistic infections, Kaposi's sarcoma, lymphoma), LAS, or ARC, who belonged to the major risk groups (homosexuals, hemophiliacs, drug users, Haitians, Africans, etc) (6, 38).

More recently, retroviruses with similar morphological and antigenic properties have been isolated in several other laboratories (39–41) from individuals of the same groups. Various names, HTLV-III (human T lymphotropic virus-III) (39) or ARV (AIDS-related virus) (41), have been given to some of these isolates, but clearly they are antigenically and molecularly related to LAV (42, 43). Thus, they appear to be variants of the same virus.

Characteristics of the Virus

LAV has the general properties of a retrovirus: (*a*) morphogenesis by budding at the cell surface (Figure 1), (*b*) envelope made of a major

glycoprotein, (*c*) density in sucrose gradient of 1.16 to 1.17, (*d*) high molecular weight RNA (70S), and (*e*) presence of a reverse transcriptase (RT) that has chromatographic properties similar to those of other retroviruses (6).

MORPHOLOGY Mature virions, 110–130 nm in diameter, have a characteristic morphology with a small dense eccentric round or bar-shaped core, depending on the section incidence (Figure 1).

Viral buds at the cell surface show a dense crescent, presumably the viral ribonucleoprotein, separated from the surface by a characteristic structured material. This organization persists in free particles, before the core's condensation. Spikes of the glycoprotein appear to be higher at the cell surface and in immature virions than in mature particles (Figure 1). Reduction of the envelope carbohydrate content might explain these differences (44).

PROTEINS Four antigenic major proteins can be detected. Three of these proteins are associated with the core: (*a*) the p13, whose basicity suggests that it is tightly bound to viral RNA; (*b*) the p18; and (*c*) the p25. They are synthesized as a unique precursor of 55 kilodaltons (kd), which is probably cleaved by the action of a virus-coded protease (45).

The fourth protein is the envelope protein, a large glycoprotein (gp) of 110 kd. Using immunoblotting techniques, it has been shown that antibodies against the gp also recognize some peptides of lower molecular weight (70, 41, and 34 kd) that are likely to be its cleavage products (44).

VIRAL GENOME The complete LAV genome has been cloned (46–48) and sequenced (43, 45, 48, 50). It is 9193 base pairs long, larger than that of most other retroviruses, except for lentiviruses.

The proviral genome structure is original (Figure 2). Besides the three classical retroviral genes (*gag*, *pol*, and *env*) and the regulatory sequences (long terminal repeats = LTR), there exist two open reading frames (*orf*) named *q* and *f*, lying respectively between *pol* and *env*, and between *env* and the LTR3′. These *orf* could code for two proteins of 22–23 kd, not yet identified.

LAV proviral DNA hybridizes under high stringency conditions with that of HTLV-III and ARV, but not with that of HTLV-I or -II, even under low stringency (46). Sequence data confirm the absence of relevant homology between LAV and HTLV-I and -II DNAs, an absence already suggested by the different morphology and the lack of cross-reactivity between viral antigens.

Thus, LAV seems to be the prototype of a second group of human retroviruses that diverged long ago from the others (51). By its morphology,

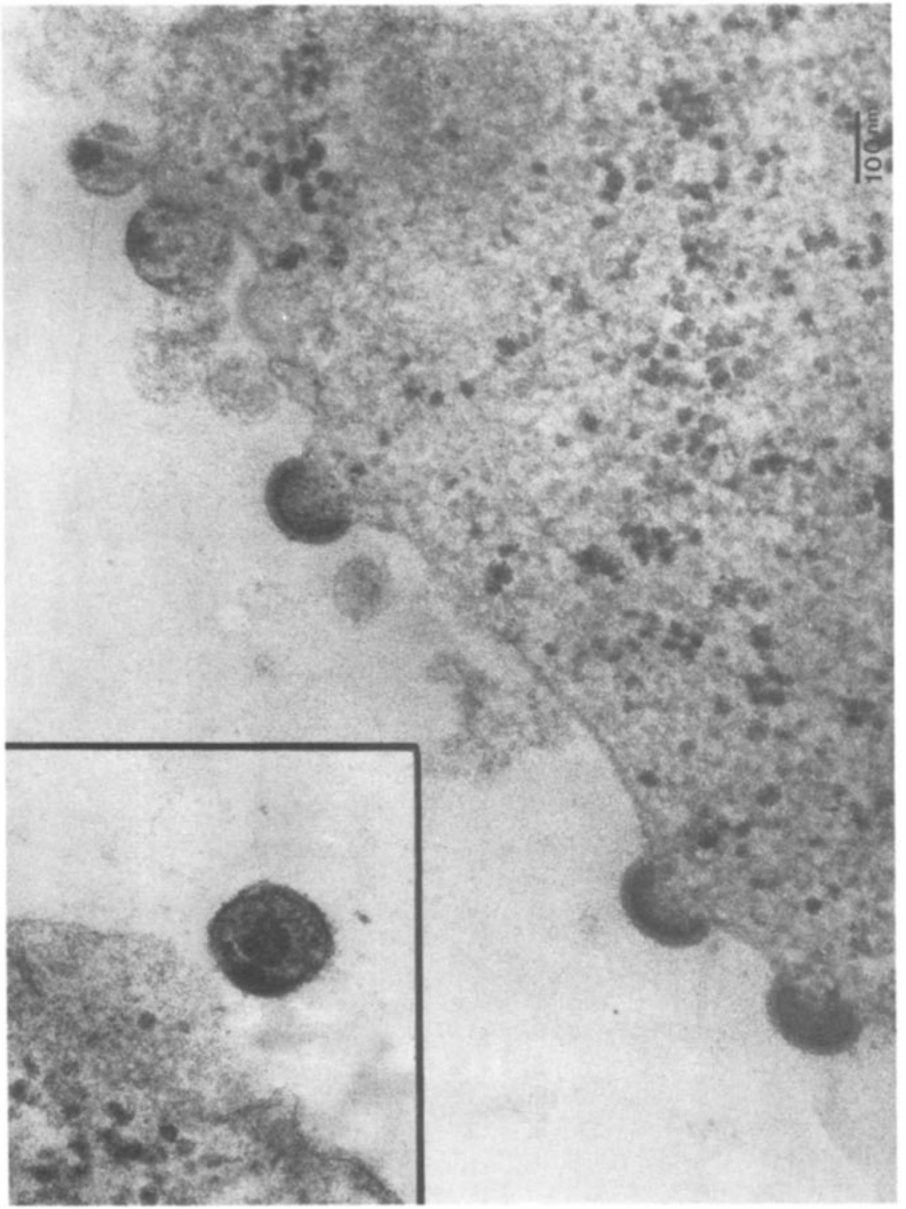

Figure 1 Electron micrograph of a section of CEM cell infected with LAV1. Note the budding particles. Insert: a typical mature virion.

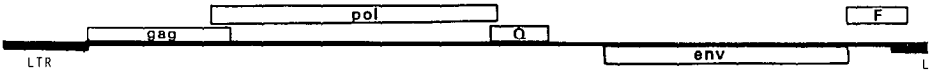

Figure 2 Diagram of the provirus structure of LAV1 (after Wain-Hobson et al (45). *q* and *f* are two extra open reading frames.

the size of its glycoprotein and of its genome, and the antigenic cross-reactivity of its major core protein (p25) to that of equine-infectious-anemia virus (EIAV) (52), it appears to be close to the subfamily of lentiviruses whose prototype is the sheep's visna virus. The genetic variation of the envelope gp of lentiviruses is well known, and it may even impair humoral responses against these viruses (53, 54). Actually, sequence comparison of different isolates indicates that the *env* gene of ARV differs by about 20% in the 3′ region from that of the LAV or HTLV-III strains, while their overall sequence difference is approximately 5% (43). Sequence comparison with more isolates is needed to evaluate the degree of variation in LAV and to determine whether there exists a small number of definite subtypes or a continuous variation within isolates even in a given infected individual. However, antigenic variations between isolates do not seem sufficient to impair detection of antibodies to viral proteins in all groups of patients. Especially, antibodies against the gp can be detected in all adult AIDS patients when the prototype strain, LAV1, is used as antigen (44).

IN VITRO BIOLOGICAL PROPERTIES OF LAV

Conditions for Optimal Virus Production

Lymphocyte activation appears necessary for virus expression and replication and, as for many other retroviruses, LAV seems to require active mitotic cells to replicate. Usually this is obtained by using phytohemagglutinin (PHA) followed by culture with a conditioned medium containing IL-2. Other T-cell mitogens such as Concanavalin A (Con A) or T- and B-cell mitogens (pokeweed mitogen, wheat germ agglutinin) (55) can replace PHA. In this respect, a toxin isolated from *Streptococcus pyogenes* group A, a preferential CD4+ mitogen (A. M. Korinec et al, submitted), has been particularly efficient for virus propagation in lymphocytes from adult normal blood donors.

In addition, stimulation by allogeneic normal lymphocytes, by allogeneic or autologous Epstein-Barr virus (EBV)–transformed B-lymphoblastoid cell lines, or even by soluble bacterial (PPD) or fungal (candidin) extracts can also be used instead of lectins for virus production.

Selective Tropism of LAV for Helper-Inducer T Lymphocytes

Some of the earliest evidence that LAV might be the cause of AIDS stemmed from the demonstration of its highly selective tropism for the very same lymphocytes that are affected in AIDS, namely the CD4+ helper-inducer T lymphocytes. This has been demonstrated in two ways (31, 56): (*a*) When T lymphocytes from an asymptomatic virus carrier (7) were separated into CD4+ and CD8+ cells by cellular affinity chromatography with the relevant monoclonal antibody, only CD4+, not CD8+, were able to express the virus upon activation (Figure 3). (*b*) In parallel experiments, only CD4+ lymphocytes from normal individuals could be productively infected in vitro with LAV after PHA activation and culture.

However, even at the peak of virus production, few CD4+ cells (<10%) express virus antigen as determined by indirect immunofluorescence staining on fixed cells with LAV-specific patients' sera or monoclonal antibodies. This finding suggests either (*a*) that most of the cells are infected but only a few are virus producers at a given time, or (*b*) that only a fraction of CD4+ cells is susceptible to LAV infection, which indicates a functional

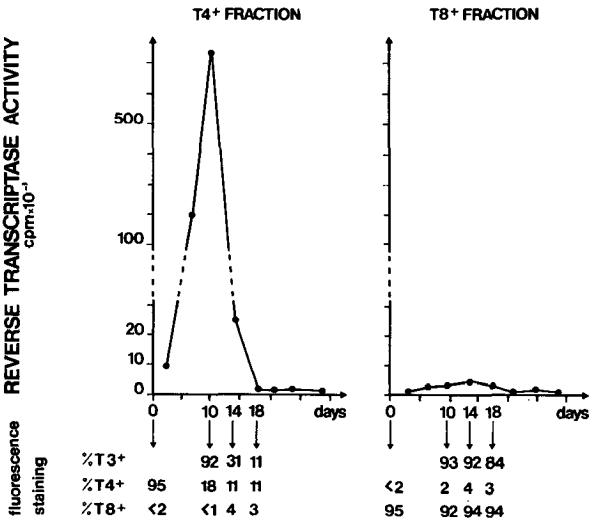

Figure 3 Virus production and phenotype of cultured lymphocyte subsets. $CD4^+$ and $CD8^+$ fractions were purified by cellular affinity chromatography (56). Reverse transcriptase activity was determined every 3 to 4 days. Cell suspension phenotypes were determined on days 0, 10, 14, and 18. (Reprinted by permission from D. Klatzmann et al. *Science* 225: 59–63. Copyright © 1984 by the AAAS.)

Table 1 Modification of activated lymphocyte markers at the time of virus replication in LAV-infected cultures[a]

Lymphocytes	% Lymphocytes expressing: CD3	CD4	CD8	DR	IL-2R[b]	RT[c] (cpm[d] × 10^{-3})
LAV(−)	96	46	54	59	27	0.4
LAV(+)	75	24	50	25	10	30.6

[a] Lymphocytes were stimulated with PHA. After 3 days, they were infected by LAV and then maintained in culture using conditioning medium containing IL-2. The experiment described here was conducted 9 days after LAV infection. LAV(−) cells were cultured under the same conditions without infection.
[b] IL-2 receptor as determined by the Tac antigen.
[c] Reverse-transcriptase.
[d] Counts per minute.

cation associated with reduced IL-2 receptor expression can be noted from the time of the peak of virus production in LAV-infected lymphocyte cultures maintained in IL-2-conditioned medium (Table 1).

Continuous Cell Lines

Some transformed lines of the T-cell lineage are permissive to the virus. They generally express the CD4 marker at their membrane (60). In this respect, clones of the CEM line, derived from a lymphoblastic leukemia of a four-year-old child, have been particularly suitable for high-titer LAV production, since most of the cells replicate the virus. Inhibition of cell multiplication, lysis of infected cells, and occurrence of giant cells can, however, also be noted in these lines.

EBV-TRANSFORMED LYMPHOBLASTOID CELL LINES Some lymphoblastoid cell lines, obtained by EBV transformation of B lymphocytes, can also support LAV replication (63). Only some cell lines are susceptible to the virus, and it is possible that ability to be infected by LAV depends on the transient expression of the CD4 molecule in some of the cells (60). The possibility that an EBV-negative cell line (BJAB) can be infected by LAV indicates that EBV by itself is not necessary for viral infection, though yield of the virus is greatly enhanced upon transformation of this line by EBV; this suggests some kind of positive interaction between the DNA virus and the retrovirus.

The possibility that EBV-transformed B cells may serve as reservoir for LAV in AIDS patients, in whom EBV reactivation is often seen, cannot be excluded. It should be stressed, however, that only a few LAV or HTLV-III strains have so far demonstrated such a tropism, perhaps because they went through adaptation during a long in vitro propagation. Nevertheless, the

biochemical and antigenic properties of the "B" LAV strain remain unchanged, as well as its selective tropism for the CD4+ subset of normal T lymphocytes (63).

CURRENTLY AVAILABLE MARKERS OF LAV INFECTION

Up to now, the most commonly available marker of LAV infection in individuals who have encountered this virus is the detection of specific antibodies directed against the major viral proteins.

Antibody Assays

Various techniques, which do not always give the same results, are available:

1. Immunofluorescence on LAV-infected fixed cells should be interpreted with caution since LAV-infected individuals often display antibodies that can react against normal lymphocyte antigens (64). Hence, proper controls have to be included in each preparation.
2. The enzyme-linked immunosorbent assay (ELISA) appears to be sensitive and specific. To avoid the pitfall of nonspecific IgG binding by lymphocyte antibodies, our group developed an ELISA with a control antigen made of a lysate from uninfected lymphocytes (65).
3. The radioimmunoprecipitation assay (RIPA) (44) uses ^{35}S-cystein metabolically labeled virus, electrophoresed in a polyacrylamide gel (SDS-PAGE) after immune complex formation with antibodies of positive sera.
4. Immunoblotting, or Western blot, is a sensitive technique that does not necessarily detect the same types of antibodies as RIPA (44). This is especially true for high molecular weight molecules, such as gp 110, which are less well transferred than smaller proteins.

Only RIPA and Western blot allow precise determination of antibody specificities to each of the core proteins, or to envelope proteins such as gp110. Nevertheless, sera that are positive with one technique are usually also positive with the others, though immunofluorescence or ELISA can sometimes give false positive results in healthy individuals.

Sero-Epidemiological Data

PATIENTS WITH AIDS AND RELATED SYNDROMES The strongest antibody responses are generally noted among patients with LAS. Of these, 90% are positive in the ELISA, most with antibodies against the viral core proteins, while some present only with anti-gp110 antibodies (44, 65).

There is a frequent decrease of the antibody response to LAV in AIDS patients during the course of the disease, and those directed to core proteins may even disappear while those against the gp110 remain detectable till the end (7, 44).

HEALTHY INDIVIDUALS BELONGING TO HIGH RISK GROUPS A high incidence of LAV seropositivity (18% in 1983, 35% in 1984) has been noted in homosexuals consulting a sexually transmitted disease (STD) clinic in Paris (65; and J. C. Gluckman, L. D. Lachiver et al, unpublished observation). Similar or even higher percentages—greater than 60%—have been found among homosexuals from London, New York, and San Francisco (68, 69). Similar figures can be observed in intravenous drug abusers (68).

Hemophiliacs are also largely seropositive: In a recent survey, 59% of French hemophiliacs heavily treated with factor VIII or IX were positive, compared to 10% of another group less heavily treated (70). Hence, viral transmission appears to occur through antihemophilic preparations containing LAV-infected plasma.

Other populations potentially at risk, such as multitransfused individuals, have not yet been systematically investigated. However, the systematic detection of LAV-seropositive blood donors will almost eliminate any risk in such patients.

GENERAL POPULATION It is quite possible that, besides known "risk groups," there exist seropositive individuals in the general population. Studies (65) that have now extended to thousands of blood donors in France indicate a 0.1–0.3% prevalence of LAV seropositivity in the French population. Similar results have been obtained in other Western countries and in the USA (71, 72).

The situation appears different in central Africa. As early as 1980, 5% of the sera of Zairan young mothers was positive (66), and similar data have been found in neighboring countries (73). This higher prevalence of LAV infection in Equatorial Africa could be due to an earlier dissemination of the virus.

Significance of LAV Seropositivity

LAV seropositivity is a marker of viral infection but this does not predict subsequent disease development. However, although the virus can frequently be isolated from the cultured lymphocytes of seropositive (but not from seronegative) individuals, it is not yet known whether LAV seropositivity is an indicator of past, latent, or productive infection. One cannot therefore clearly specify those individuals who are able actually to transmit the virus by intimate contact or blood donation.

Our current hypothesis is that antibodies against core proteins reflect recent expression of the whole virus followed by lysis of virions or infected

cells, whereas antibodies against the gp110 are determined by a constant expression of the latter at the infected lymphocyte surface even in the absence of whole virus expression. Only new techniques, that allow direct and easy detection of LAV itself or its constituents in biological fluids and lymphocytes will allow a more complete understanding of the natural history of LAV infection. It will perhaps appear then that, in some instances, one can be a virus carrier without having developed antibodies to LAV (74).

A PATHOPHYSIOLOGICAL MODEL OF AIDS

The etiological role of LAV in AIDS and related syndromes is supported by many observations: (*a*) Up to now, isolates have been almost exclusively obtained from AIDS, LAS, and ARC patients, their sexual contacts, or individuals at risk. (*b*) Similarly, serological data indicate the prevalence of LAV infection in AIDS and related syndrome patients as in healthy individuals at risk for these diseases. (*c*) Short of the so-called "Koch postulate," the best evidence of causal relationship between LAV and AIDS comes from documented cases of accidental transmission of the virus and the disease by blood transfusions (40, 75–77). (*d*) Finally, LAV's biological properties, especially its CD4+ lymphocyte tropism and its cytopathic effect, are exactly those that would be expected from the AIDS etiologic agent.

It is nevertheless obvious that LAV infection is most often asymptomatic and that it can remain latent, at least for years, in some individuals. The cofactors that contribute to the subsequent development of AIDS are presently unknown, but it is now possible to delineate some likely mechanisms of the disease pathogeny.

Individual Susceptibility to Virus Infection

The hypothesis that a particular immunologic "terrain" might influence the host's response to LAV and predispose to the development of disease stems from the observation that immune abnormalities can be noted, even in the absence of LAV infection markers, in individuals from most, if not all, groups at risk for AIDS.

For example, we recently conducted a study of 31 high risk male homosexuals attending a STD clinic who were of age and life style comparable to the AIDS patients. Results were compared to those of a control group of 35 heterosexual men attending the same clinic, who were in the same age range and presented with a similar pattern of STD. Antibodies to LAV were detected in 11 (35%) of the homosexuals and in none of the heterosexuals. These latter had, on the average, slightly reduced delayed-type hypersensitivity (DTH) responses as compared to a normal control

population ($P < 5 \times 10^{-2}$), but their lymphocyte and T-cell counts were in the normal range. Comparatively, the homosexuals had significantly reduced DTH reactivity, decreased $CD4^+$-cell numbers, and lower $CD4^+/CD8^+$ ratios (Table 2). These abnormalities could not be associated with the existence of LAV antibodies, although mean values were always lower in LAV-positive individuals. Moreover decreased DTH and low $CD4^+$ counts could be observed in seronegative as well as in seropositive subjects: Response to <1 antigen occurred in 6/20 vs 5/11, and $CD4^+$ counts <500 μl (23) in 7/20 vs 4/11, respectively. In addition, the LAV-negative homosexuals still significantly differed from the heterosexuals with respect to these parameters. These results indicate that LAV, which is expected to affect the cellular immune system because of its tropism, is but one of the causes of immune test abnormalities in homosexuals. Other factors are possibly also involved, particularly since similar disturbances have been described in a variety of infections (78), especially with viruses of the Herpes group (79–80), which are known to occur with high incidence in this population (81). However, we did not note any relationship with the current bacterial or viral STD presented by 26 (74%) of the heterosexuals and 12 (36%) of the homosexuals. Altogether, reduced DTH reactivity or low $CD4^+$ cell counts do not appear to be relevant indicators of LAV infection, at least in symptomless homosexuals, and other immunological markers have yet to be defined.

Such a situation also appears to be true for hemophiliacs (82, 83) (probably because of repeated injections of foreign proteins) as well as for Africans or Haitians, who often present with immunodepressing diseases (tuberculosis, malaria) (66).

Genetic Background

The occurrence of autoimmune diseases, such as thrombocytopenia (84, 85), and the presence of immune complexes (22) and/or of antilymphocyte antibodies (64), is frequently observed in individuals at risk for AIDS. It is thus possible that, in full-blown AIDS, immune deficiency first induced by LAV is further exaggerated by as yet unidentified immune processes.

Such autoimmune reaction might then well depend on the host's genetic constitution and especially on his HLA background.

Antigenic Stimuli

In most cases, LAV primary infection is inapparent. Only a small number of mature $CD4^+$ lymphocytes in the blood or lymph nodes, or precursors in the bone marrow (25), integrate the proviral genome. It is only when these infected lymphocytes are activated during viral, bacterial, or fungal infections, or by contact with allogeneic cells (lymphocytes for example)

Table 2 Immunological studies in homosexual men without symptoms of AIDS or lymphadenopathy syndrome and in a control group of heterosexuals

		DTH[a]:		Cell counts per μl[c]:			
	N	Mean number of + reactions	Lymphocytes per μl	CD3+	CD4+	CD8+	$CD4^+/CD8^+$ ratio
Populations							
Homosexuals (Hm)	31	2.00 ± 1.32	2070 ± 650	1415 ± 500	670 ± 355	810 ± 330	0.90 ± 0.50
LAV positive (+)	11	1.45 ± 1.13	1970 ± 605	1370 ± 385	575 ± 295	835 ± 440	0.75 ± 0.45
LAV negative (−)	20	2.30 ± 1.34	2125 ± 685	1440 ± 465	720 ± 380	795 ± 270	1.00 ± 0.55
Heterosexuals (Ht)	35	3.06 ± 1.08	2190 ± 525	1680 ± 470	1095 ± 315	670 ± 270	1.80 ± 0.70
Statistical significance							
Hm vs Ht		$P < 10^3$	NS[b]	NS	$P < 10^{-6}$	NS	$P < 10^{-8}$
LAV (−) vs LAV (+)		NS	NS	NS	NS	NS	NS
LAV (−) vs Ht		$P < 5 \times 10^{-2}$	NS	NS	$P < 10^{-4}$	NS	$P < 10^{-4}$

[a] Delayed-type hypersensitivity (DTH) was assessed by the MULTITEST® (Merieux, France), a device that allows investigation of the cutaneous response to seven different antigens. Results are expressed as mean ± SD.

[b] NS = not significant.

[c] Peripheral-blood T-cell populations were measured by indirect immunofluorescence with monoclonal antibodies.

such as are present in semen (86), that they would actively replicate the virus. Indeed frequent infections or contacts with allogeneic cells or proteins are common in homosexuals and hemophiliacs at least.

LAV infection would then spread to other helper-inducer T lymphocytes whose progressive destruction would lead to severe and irreversible immune deficiency. In some patients, the disease could be limited to the lymph nodes by active immune defense reactions, not yet described, which would thus induce lymph node hyperplasia.

Multiple LAV Infections

LAV diffusion might also be related to multiple exposures to this virus. Indeed, LAV infection might follow the same pattern as that reported for lentiviruses: sheep can be infected repeatedly with several visna virus strains, each persisting for a long time in the host; the equine infectious anemia virus seems to undergo genetic changes at the level of its envelope gp, thereby escaping the host's neutralizing antibody response (87, 88).

No data presently available show whether such variations are possible for LAV, but it is known from sequence comparison of several isolates that the *env* gene displays great genetic variation compared to other viral genes.

Synergistic Effects of Other Virus Infections

We have already indicated that EBV-infected B lymphocytes might serve as a reservoir for LAV when $CD4^+$ cells have all but disappeared. EBV could also be at the origin of polyclonal B-cell activation, a characteristic feature of LAV-related diseases. This activation could also be the consequence of a suppressor-cell defect in relation with the deficiency of suppressor-inducers present in the $CD4^+$ T-lymphocyte subset (89).

Finally, DNA viruses such as CMV or hepatitis B virus that also replicate in T cells may also have synergistic effects on LAV replication.

CONCLUSION

There presently exists a consistent pathophysiological model for AIDS development. Testing the various hypotheses mentioned here is of primary importance, since this will help to delineate future therapeutic and preventive strategies applicable to LAV-infected individuals.

This goal will ultimately come from cooperative research programs involving clinicians, immunologists, virologists, and molecular biologists.

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CONTENTS

Ann. Rev. Immunol. 1986. 4 : 119–45

EFFECTOR MECHANISMS IN ALLOGRAFT REJECTION

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GENERAL INTRODUCTION

This review is divided into two parts. The first of these on the mechanism of allograft rejection concerns studies made with experimental animals and the second, the observations made in clinical transplantation.

The experimental data relate mainly to the effector mechanisms of graft rejection but, because it is impossible to consider these without also considering how these effector mechanisms are called into play, a brief outline of present knowledge of the induction of the rejection process is also included.

In humans, most immunological and histological observations have been made of patients with renal allografts, which are primarily vascularized tissue allografts where the rejection reaction has been modified by immunosuppressive therapy. Despite these differences from much of the experimental animal data, the interpretation of the human data is dependent on the experimental work and seems likely to remain so for some time. The need to understand the mechanisms by which an organ allograft is rejected in humans is essential for the design of better immunosuppressive therapy.

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EXPERIMENTAL

The study of the immunological responses of laboratory animals to tissue or organ grafts from genetically dissimilar donors continues to be an active field of research although it is almost 40 years since the discovery of the major histocompatibility complex (MHC) (1). Allogeneic and xenogeneic grafts evoke a wide range of immunological effector mechanisms, both aggressive and, in some instances, tolerogenic; this fact together with the clinical importance of organ transplantation has ensured continued interest in this subject.

The aim of this section is to review present knowledge of how grafts are rejected in experimental animals and to indicate where this knowledge is inadequate.

Most of the data discussed are concerned with the rejection of skin or organ grafts, but some papers on the rejection of tumor grafts are also described. The rejection of bone marrow or lymphoid cell grafts is not considered.

THE CLASSICAL SCHEME

Solid tissue or organ grafts are apparently rejected by a process conveniently divided into three stages. First, antigenic material is released by the graft into the draining lymphatics (afferent stage); it then activates the immune reaction in the recipient lymph nodes (central stage). Finally, cellular and humoral effector mechanisms, generated by this reaction, leave the nodes via the efferent lymphatics, enter the blood stream, are thence delivered to the graft, and bring about its destruction (efferent stage) (2). This scheme is supported by the findings that grafts placed into sites that have no lymphatic drainage (3) or that do not receive a blood supply (4) enjoy prolonged survival, as do grafts placed in animals deficient in lymphocytes (5, 6). With the discovery of the role of the thymus in lymphocyte differentiation and the finding that congenitally athymic mice do not reject allografts or even xenografts, researchers soon recognized that the T lymphocyte played the essential role in the rejection process (5, 6). A similar conclusion was drawn with respect to tumor allografts (7).

Recent work has made significant contributions to the further understanding of all three phases of this rejection process.

THE AFFERENT ARM

The antigenic material that leaves an organ graft and stimulates the draining node has been identified almost conclusively as a bone marrow–derived dendritic cell. Earlier experiments showed that rat renal allografts

from bone marrow chimeras often survived indefinitely in normal hosts if the bone marrow–derived cells in the grafts were syngeneic with the graft recipients (8). These observations were followed by the demonstration that allogeneic rat kidneys that had resided for some time in an immunosuppressed host could often be transferred to a *normal* secondary host, syngeneic with the first, without rejection (9, 10). In addition, researchers found that thyroid allografts, depleted of bone marrow–derived passenger cells by prior in vitro culture, also enjoyed prolonged survival in unsuppressed recipients (11). These experiments have led to the idea that organ allografts are immunogenic by virtue of their content of a migrant population of "passenger leukocytes," a concept first introduced almost 30 years ago to explain tumor allograft rejection (12).

The identification of the passenger leukocyte resulted from the discovery that cells with striking dendritic morphology, recovered from mouse spleen (13) and rat afferent lymph (14), expressed large quantities of class-II MHC antigens and were potent stimulators of the mixed leukocyte reaction. When relatively small numbers of such cells, recovered from rat afferent lymph, were injected into rats receiving passenger leukocyte–depleted kidney allografts syngeneic with the dendritic cell donors, this caused rejection of the organ grafts (10), whereas injection of lymphoid cells did not. Finally, using immunofluorescence to detect class-II MHC antigen-positive cells in cryostat sections (15), researchers showed that dendritic cells were widespread in mammalian tissues (absent only from brain) and were likely therefore to play a significant role in almost all studies of organ transplantation.

The potential clinical importance of dendritic cells in stimulating the rejection of organ grafts has been stressed by the finding that pancreatic islet tissue, cultured in vitro in conditions believed to destroy passenger leukocytes, can sometimes be transplanted successfully from allogeneic and even xenogeneic (rat) donors into recipient mice without chronic immunosuppression, whereas uncultured grafts are rejected (16).

ARE DONOR-STRAIN DENDRITIC CELLS ESSENTIAL FOR ALLOGRAFT REJECTION?

There are exceptions to the rule that allograft survival in rodents is assured if dendritic cells are removed from the graft before transplantation. When rat renal allografts, depleted of dendritic cells, are transplanted into non-immunosuppressed recipients, the subsequent fate of the grafts depends on the donor/host strain combination used. In some of these combinations, grafts survive indefinitely, while in others rejection occurs with a tempo little different from that seen when normal allografts are transplanted (9, 17). Furthermore, passenger leukocyte–depleted rat pancreatic islets are

usually rejected in allogeneic recipients (18), and similar results are found in some mouse strain combinations (19). Finally allografts of neuronal tissue, which contain no demonstrable dendritic cells, may undergo rejection when placed in the third ventricle of recipient rats—in this case rejection occurs with a tissue lacking dendritic cells and a transplantation site lacking a lymphatic drainage! In this instance, however, the rejection is slow (20).

There are two possible explanations for these results. Either dendritic cells are not unique in their ability to activate host inducer/helper T cells; alternatively, graft transplantation antigens can be processed by host dendritic cells, as conventional antigens are believed to be, and presented to host T cells in this way. There is evidence for both of these possibilities, and they are not mutually exclusive. Vascular endothelium can be induced to express class-II MHC antigens in rodents (21, 22) [and is constitutively expressed on this tissue in humans (23, 24)]; it has also been shown capable of inducing mixed leukocyte culture (MLC) reactivity in vitro (25). Additionally, experiments designed to test the ability of recipient mice to process both major and minor transplantation antigens and to present them to the recipients' inducer/helper-T cells have produced positive results (26, 27). These findings clearly call into question the suggestion that dendritic cell depletion of organ allografts will inevitably result in prolonged graft survival, and species differences in particular may have a profound effect on the outcome (28).

CENTRAL EVENTS AND EFFECTOR MECHANISMS

The fate of dendritic cells entering a lymph node via the afferent lymphatics is not known, but bone marrow–derived cells with similar morphology that express similarly high levels of class-II MHC antigen can be demonstrated in the thymus-dependent areas of the node (29). Bearing in mind that dendritic cells stimulate vigorous mixed leukocyte culture responses, we may anticipate that if such cells enter the T-dependent areas of allogeneic hosts, prompt T-cell activation will ensue. Cellular interactions occurring in antigen-reactive lymph nodes are difficult to study in situ, but in vitro experiments have demonstrated a central role for the inducer/helper T cell (T_h) in regulating the immune response. As Figure 1 shows, T_h cells are implicated in activating a number of effector mechanisms, any one of which might, in principle, bring about destruction of allografts.

ROLE OF DELAYED-TYPE HYPERSENSITIVITY AND CYTOTOXIC T CELLS IN GRAFT REJECTION

Noting the histological similarities between the cell infiltrates in skin allografts and those in delayed-type hypersensitivity (DTH) reactions,

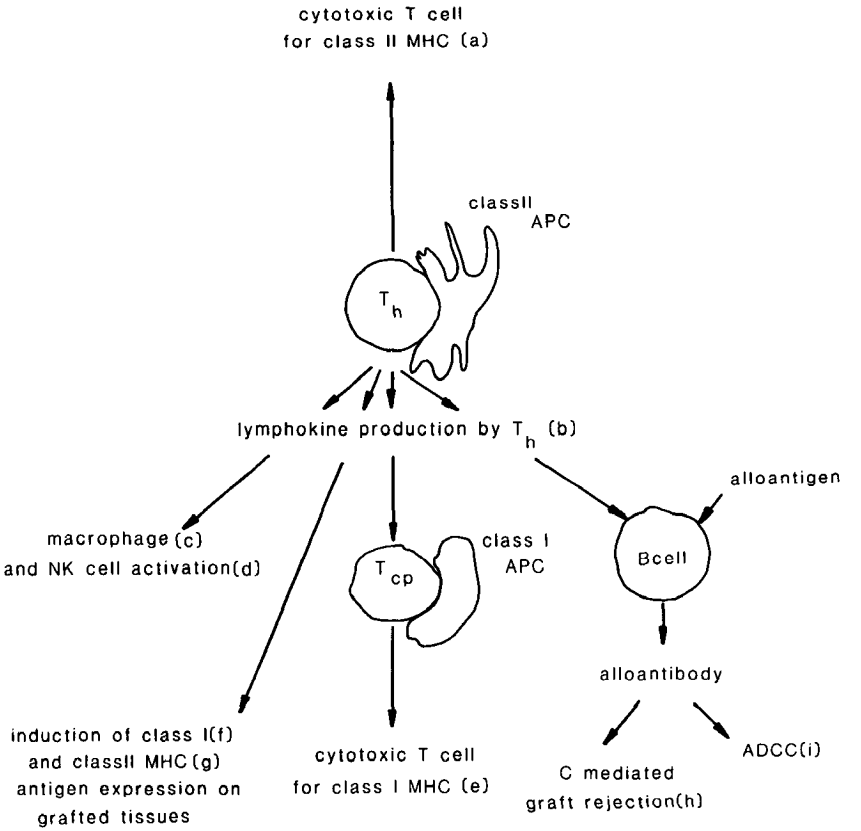

Figure 1 The Central Role of Inducer/Helper T Cells in Allograft Rejection. The diagram illustrates, in broad outline only, the role played by inducer/helper T cells in promoting the activation, proliferation, and differentiation of different effector mechanisms involved in immune reactions. Further details are contained in the references quoted. The diagram also draws attention to the ability of inducer/helper T cells to induce or enhance the expression of MHC antigens on nonlymphoid tissue such as the vascular endothelium of allografts. The letters (a)–(i) in the figure correspond to the following numbers in the references listed at the end of this article. (a)—44; (b)—71–73; (c)—74; (d)—75; (e)—76; (f)—21; (g)—21–23; (h)—77; (i)—78. Abbreviations: APC, antigen-presenting cell; NK, natural killer; T_h, inducer/helper cell; T_{cp}, cytotoxic T-cell precursor; C, complement; ADCC, antibody-dependent cell-mediated cytotoxicity.

earlier workers suggested that allografts were rejected by a mechanism similar to that mediating DTH (30). With the discovery of cytotoxic T cells (Tc) (31) this earlier view was largely discarded (32, 33), but it has recently been revived because of observations of the capacity of different T-cell subsets to restore the ability of T cell–deprived rodents to reject skin and organ allografts. Experiments in both rats (34) and mice (35) have shown

that animals made T cell–deficient by adult thymectomy, lethal irradiation, and bone marrow reconstitution (ATXBM animals) do not reject skin allografts, but their ability to do so can be restored by inocula of syngeneic T cells of inducer/helper phenotype, i.e. Lyt 1^+2^- cells in the mouse and W3/25$^+$ OX-8$^-$ cells in the rat. Cells that mediate DTH reactions are known to be of inducer/helper phenotype, and the above results have been interpreted as supporting the view that allografts are destroyed by a mechanism essentially the same as that responsible for the tissue damage that accompanies DTH reactions to tuberculin (35). Doubt must be cast on this conclusion for two reasons. First, the T-cell deficiency in ATXBM rats and mice and in congenitally athymic mice appears to be confined to the inducer/helper–T cell subset. That such animals possess the precursors of cytotoxic T cells is shown by the fact that lymphocytes from these animals will differentiate, both in vitro and in vivo, into specific cytotoxic T cells if provided with the appropriate inductive signals from inducer/helper T cells (36–39). Significantly, it has recently been shown that cytotoxic T cells can be recovered from the spleens of ATXBM mice receiving skin allografts and inocula of inducer/helper-T cells (40), and cells of cytotoxic-T-cell phenotype have been found in skin allografts of rats similarly treated (34). In an attempt to minimize any host contribution to the rejection process, researchers have subjected animals to sublethal irradiation at the time of grafting (40, 41, 42, 43). Results from these experiments are essentially the same as those using ATXBM animals in that T cells with inducer/helper phenotype, when transferred to the irradiated hosts, cause allograft rejection to occur with a similar tempo. In some of these experiments, the restorative inocula have been contaminated with cells of the classical cytotoxic phenotype since specific cytotoxic T cells, derived from the donor inocula, have been isolated from the spleens of mice subject to this protocol (40). Similarly cells with cytotoxic phenotype have been found in heart grafts of acutely irradiated rats given inducer/helper T cells (43). However, not all attempts to detect classical cytotoxic T cells in experiments of this type have proved successful (40, 42). Possible explanations for these negative results are discussed later in this review.

The second problem in ascribing graft rejection to a DTH-like mechanism arises because it has been shown that cytotoxic T cells with specificity for class-II MHC antigens arise from precursors with inducer/helper phenotype (44), and cytotoxic–T cell clones expressing this phenotype have been isolated (45). Curiously, although some show specificity for class-II MHC antigens (45), others recognize class I (46). The existence of cytotoxic–T cell precursors with inducer/helper phenotype clearly poses problems for the argument that cytotoxic T cells are not involved in the graft rejection observed in these experiments. However, attempts to recover cytotoxic T

cells for class-II positive targets, from organ grafts in sublethally irradiated rats given cells of inducer/helper phenotype, have yielded negative results (47). Further discussion of these findings will be deferred until later in this review.

INDUCIBILITY OF CLASS-II MHC ANTIGENS ON ALLOGRAFTS

One potential argument against the suggestion that cytotoxic T cells specific for class-II positive targets may be important in allograft rejection is that the tissue distribution of such antigens is rather limited. Thus, for example, although rat cardiac allografts, in sublethally irradiated hosts given primed T cells of inducer/helper phenotype, undergo rejection (42), class-II MHC antigen in normal rat heart is found only on the bone marrow–derived dendritic cells (21). However, it is well established that class-II MHC antigen expression can be induced on a variety of allograft tissues including the vascular endothelium and myocytes of rat heart grafts (21, 22, 48). Induced class-II antigen expression can be important in allograft rejection. Thus, mouse skin grafts exchanged between recombinant mice differing only for MHC class-II antigens are rejected, although the only class-II positive tissue in *normal* mouse skin is comprised of the Langerhans cells, and these cells are not targets for the rejection process (49). In these skin grafts the activation of host T cells, by Langerhans cells of the graft, may result in lymphokine production and consequent induction of class II-MHC antigens on the graft vasculature. These blood vessels may then become the target of the rejection mechanism (22, 50).

EVIDENCE THAT CYTOTOXIC T CELLS CAN DESTROY ALLOGRAFTS

There are several lines of evidence that support the view that cytotoxic T cells are able to mediate allograft rejection. An early experiment of Mintz & Silvers (51) suggested that graft rejection was mediated by a process that destroyed individual allogeneic cells rather than by one that caused tissue damage nonspecifically as a bystander effect. When skin grafts from allophenic mice, produced by the fusion of blastomeres of two different H-2 strains, were transferred to recipients of one of the parental strains, melanoblasts and hair follicles that expressed the same H-2 antigens as the host survived, while those expressing the foreign H-2 antigens were destroyed. Similarly, no bystander cytotoxicity was observed when two different tumors were mixed and introduced, together with lymphoid cells preimmunized to only one of the tumors, into the peritoneal cavity of

irradiated mice. In this experiment only the tumor that was the specific target for the sensitized cells was rejected (7).

While mitigating against DTH as the effector mechanism, these experiments could not directly implicate cytotoxic T cells. However, when mouse lymphoid cells, sensitized against a tumor allograft, were separated into adherent and nonadherent fractions by adsorption onto specific fibroblast monolayers, it was found that the nonadherent fraction was less effective in retarding tumor growth in vivo and lysing tumor cells in vitro, whereas both these functions were enhanced in the adherent population (52).

More recently, a cytotoxic–T cell clone has been shown to eliminate susceptible tumor cell grafts in mice (53), and another cytotoxic clone, to cause local tissue necrosis when injected intradermally into mice expressing the minor, skin-specific tissue antigen to which the cytotoxic–T cell clone was directed (54). The results with these cloned effector cells, although unambiguously demonstrating the in vivo cytotoxic capacity of the clones, are somewhat artificial, as the clones were generated in vitro before their in vivo function was assayed. Studies on the T-cell requirement for the rejection of heart allografts in sublethally irradiated rats, where host and donor were incompatible only for class-I MHC antigens, have shown that graft rejection occurs. This is true, however, only if the restorative T-cell inoculum contains cells that are $W3/25^-$, $OX\text{-}8^+$, i.e. of the phenotype of cytotoxic T cells that recognize class-I MHC antigens (55). Similar results have been obtained in mice where studies have shown an essential role for cytotoxic T cells in the rejection of H-2D-incompatible split-heart grafts (56) and of pancreatic islet grafts (which express only class I MHC antigens) (57).

Less conclusive, but suggestive, evidence exists that cytotoxic T cells play a role in organ allograft rejection in rats. Infiltrating mononuclear cells harvested from rejecting renal allografts have been compared to those from healthy allografts in cyclosporine A–treated animals to determine their ability to lyse ^{51}chromium-labelled target cells. Both mononuclear cell populations kill NK cell–susceptible targets, but only those from rejecting allografts kill Con-A blasts of graft donor strain. These latter cells are susceptible to cytotoxic T cell–mediated destruction but are not lysed by NK cells. While supporting the conclusion that cytotoxic T cells are involved in allograft rejection, this experiment also demonstrates that apparently healthy allografts can contain large numbers of cells with NK activity. This finding strongly suggests that these cells play no significant role in organ allograft rejection in rodents (58).

Further evidence for a role for cytotoxic T cells in allograft rejection derives from experiments in which monoclonal antibodies to inducer/helper– and cytotoxic–T cell subsets have been used to study the mechanism of skin allograft rejection in adult thymectomized mice. The

results indicate that, as expected, antibody to the inducer/helper subset greatly prolonged allograft survival, but antibody to the T-cell subset containing precursors for cytotoxic T cells specific for class-I MHC antigens (Lyt 2^+ cells) also increased graft survival time in some experiments (59). A similar result has been obtained with minor histoincompatible skin allografts in rats where graft survival on sublethally irradiated animals is longer on recipients given restorative inocula depleted of cytotoxic–T cell precursors than it is on recipients given undepleted inocula (44).

CAN ALL ALLOGRAFT REJECTION IN EXPERIMENTAL ANIMALS BE ATTRIBUTED TO CYTOTOXIC T CELLS?

A number of experiments challenge the idea that, in rodents, cytotoxic T cells are essential for allograft rejection to occur. As noted above, sublethally irradiated rats reject kidney and heart allografts when injected with T cells of inducer/helper phenotype, but in several of these experiments cytotoxic T cells cannot be recovered from these animals (40, 42, 47). Similarly, in certain mouse strains, female mice will reject male skin grafts by virtue of the male specific H-Y antigen expressed on these grafts, but no H-Y-specific, cytotoxic T cells can be demonstrated in vitro. Conversely, in other mouse strains, no rejection of male skin grafts occurs despite the fact that H-Y-specific cytotoxic T cells can be generated (60). These results have led to the suggestion that cytotoxic T cells appeared to be neither necessary nor sufficient for the rejection of syngeneic male skin by female mice.

All these experiments pose severe, but perhaps not insurmountable, problems for the idea that cytotoxic T cells play an essential role in graft rejection in rodents. With regard to the rat organ graft data, it should be noted that in vitro cytotoxicity assays have rather high background levels of ^{51}Cr release that make it impossible to detect any low levels of specific cytotoxicity that may be present. Thus, the inability to detect cytotoxic T cells among those harvested from these grafts might be interpreted to imply that relatively few of these cells are needed to cause allograft destruction. In this context, note that relatively few cloned cytotoxic T cells are required to produce local tissue necrosis when injected intradermally into the appropriate mouse strain (54). The data on the rejection of male skin grafts by female mice pose a rather different problem in that, as noted, there appears to be no correlation between graft rejection and cytotoxic–T cell production in vitro. Interpretation of these findings is complicated by the fact that the H-Y antigen has not been biochemically characterized and could, in principle, comprise a number of antigens, some of which are present on skin but not on the target cells used in the in vitro cytotoxicity assays. Indeed,

there is evidence that the H-Y antigen is immunologically complex (61). Those working on the rejection of male grafts by female mice suggest that the cytotoxic T cells demonstrated in vitro may not be specific for the appropriate antigen (60). Cytotoxic T cells for H-Y, restricted to class-II MHC antigens, may play a role in these skin graft experiments, since class-II antigens may be induced on the vasculature of skin allografts in the mouse (22).

EFFECTOR MECHANISMS OTHER THAN CYTOTOXIC T CELLS AND DTH

In species other than rodents clear evidence demonstrates the importance of rejection mechanisms other than cytotoxic T cells and DTH. Thus, it is well established that hyperacute kidney rejection in humans is mediated by preformed antibody (see below). However, attempts to obtain rejection of skin (62) or kidney (63) allografts in rodents by this process have proved unsuccessful in all but a few cases (64), possibly because the lytic capacity of rodent complement is less than that of human. Paradoxically the administration of alloantibody to rodents at the time of grafting often leads to improved graft survival, a phenomenon known as enhancement (65), and it is generally accepted that antibody per se does not mediate allograft rejection in these experimental animals. However, it is less easy to exclude antibody-dependent, cell-mediated cytotoxicity (ADCC) as an effector mechanism in allograft rejection in rodents. If the accumulation of cells mediating ADCC in an allograft is dependent on the presence of inducer/helper T cells then when antibody is passively transferred to T cell–deprived rats bearing skin allografts, the inability of the antibody to bring about graft rejection need not imply that ADCC plays no role in the rejection process. However, in a number of experimental systems no correlation exists between alloantibody synthesis and graft rejection. Though they may be producing anti-H-Y antibody, female mice may fail to reject syngeneic male skin (66), while skin grafts from H-2 mutants may be rejected on wild type recipients with no detectable antibody production (67). No evidence exists that ADCC plays an essential role in experimental allograft rejection. The possibility that it may, under certain circumstances, augment the rejection reaction cannot be excluded (65).

VARIATIONS ON THE CLASSICAL SCHEME—PERIPHERAL SENSITIZATION

In the classical scheme of allograft rejection already described, the lymphatic connection between the graft and the local lymph nodes plays an

essential part. There is evidence, however, that both skin (68) and organ (69) allografts deprived of a lymphatic drainge are susceptible to rejection. In these circumstances it is believed that lymphocytes become activated to graft antigens as they pass through the graft—a mechanism that has been termed peripheral sensitization (68). This process is evidently less efficient than the central one that occurs when lymphatic drainage is intact, as the kinetics of rejection are considerably different.

CONCLUSIONS ON EXPERIMENTAL RESULTS

The most controversial topic concerning effector mechanisms of allograft rejection in experimental animals is the contribution made by specific cytotoxic T cells and by delayed-type hypersensitivity reactions. The suggestion that cytotoxic T cells, rather than DTH, can mediate graft rejection receives strong support from a number of experiments already described. The evidence may be summarized as follows:

1. Cytotoxic T cells, cloned in vitro, can cause the rejection of allogeneic tumor cells in immunosuppressed hosts (53).
2. Cloned cytotoxic T cells, injected intradermally into mice, produce local tissue necrosis, but only in those strains expressing the minor H antigen to which the cloned cells are reactive (54).
3. Tissue destruction of skin allografts derived from allophenic mice shows exquisite specificity in that cells expressing H-2 antigens allogeneic to the host are destroyed while those expressing syngeneic H-2 antigens are not (51). Similar target specificity has been shown in the rejection of tumor cells (7).
4. The ability of immunosuppressed rats to reject class-I MHC antigen-incompatible heart grafts is restored by inocula of recipient strain lymphocytes containing cytotoxic–T cell precursors, but not by inocula depleted of these cells (55, 56); cytotoxic T cells have been implicated in pancreatic islet allograft rejection (57).
5. Whereas both specific and nonspecific (natural killer) cytotoxic cells can be harvested from rat renal allografts undergoing unmodified rejection, only nonspecific effector cells can be recovered from healthy grafts in cyclosporine A–treated hosts (58).
6. Sublethally irradiated rats bearing minor-H-incompatible skin grafts reject these when injected with unfractionated inocula of recipient strain lymphocytes, but rejection is delayed if these inocula are depleted of cytotoxic–T cell precursors (44).

Despite this impressive list, there are some difficulties in ascribing

rejection of all solid tissue grafts in experimental animals to cytotoxic T cells. These exceptions are:

1. MHC-incompatible skin and organ grafts and experimental tumors in immunosuppressed rodents are rejected if the graft-bearing hosts are injected with syngeneic lymphocytes of inducer/helper phenotype (34, 35, 42, 43, 47, 70).
2. Female mice of different inbred strains show variation in their ability to reject syngeneic male skin. This variation does not correlate with the ability of these different strains to generate, in vitro, cytotoxic T cells against the male-specific H-Y antigen (60).

Explanations for these largely negative findings have been proposed in the main text, but in some instances their validity remains to be established. One major difficulty in deciding whether grafts are destroyed by a DTH-like mechanism is that there is no in vitro assay for it. Thus, most evidence that grafts can be rejected in this way derives from attempts to exclude all other possibilities. The problems inherent in this approach are self-evident.

An attempt has been made in this review to reinstate the cytotoxic T cell as an effector of graft rejection. The evidence is particularly compelling for class-I MHC incompatible grafts. This need not imply that other mechanisms, including DTH, are not equally important at least in some instances, but definitive proof is difficult to establish. Finally it should be noted that, whereas tissue damage mediated by DTH in rodents is minimal, this is not the case in humans where, for example, massive tissue necrosis may occur as a consequence of the host's immune response to infection with mycobacteria. Thus, DTH may play a more important role in clinical organ graft rejection than it does in experimental animals.

Despite the many remaining uncertainties about the mechanism of allograft rejection, almost all workers agree that inducer/helper T cells play an essential role. Any immunosuppressive regime that directly or indirectly interferes with activation of these cells is likely to prevent rejection taking place.

HUMAN STUDIES OF ALLOGRAFT REJECTION—GENERAL OBSERVATIONS

Following the observations by Gibson & Medawar that skin allografts in burn patients underwent rejection (79), most of the information about the effector mechanisms of allograft rejection in the human has been based on studies of patients with a renal transplant, i.e. a vascularized organ allograft. Although interpretation of the observations made in humans have been very dependent on the knowledge acquired from animal models discussed in the previous section, there are several essential differences

between the two: (*a*) In clinical practice the graft is between members of an outbred population, and hence the disparity in terms of major and minor histocompatibility differences is less well defined than in rodent models; (*b*) in humans the rejection reaction will always be modified by some form of immunosuppressive therapy; (*c*) much of the experimental data in animals until recently has been obtained from skin allograft models, whereas in human transplantation most of the data has been obtained from primarily vascularized renal allografts.

Most of the earlier observations of rejection of a human kidney were confined to descriptions of the histological changes in the graft itself, initially after removal of a rejected kidney and later on from Trucut needle biopsies of the graft in situ. Then attempts were made to detect antibodies to histocompatibility antigens, and finally assays were developed for the study of cellular immunity in graft recipients. The histological studies were rather limited in their ability to determine mechanisms of rejection; until recently they were dependent on a description of the morphological changes seen in the graft. But now monoclonal antibodies detecting various leucocyte subpopulations are being used to define more precisely the nature of the cellular infiltrate during rejection.

Furthermore, in part because of the modification of the immune response to a graft by immunosuppression, several types of rejection have been defined in man, based on their time of presentation after transplantation, but no doubt reflecting different effector mechanisms (80):

1. Hyperacute rejection occurs in the first 24 hr after transplantation and is almost always antibody mediated. It is seen in a recipient sensitized against donor histocompatibility antigens.
2. Accelerated rejection occurs in the first 5 days and is considered to be the equivalent of a second-set reaction in the experimental animal. This type of rejection may be mediated by cells or antibody or both.
3. Acute rejection occurs in the first 3 months (usually in the first few weeks) and is the equivalent, albeit modified by immunosuppression, of the normal first-set type of response seen in the unmodified experimental animal; again, it is mediated presumably by cells and antibody.
4. Chronic rejection occurs at any later time after transplantation and is generally associated with a rather insidious decline in renal function, accompanied by somewhat typical vascular changes in the graft itself; it may be due to low-grade cellular and humoral mechanisms of rejection or merely be the aftermath of severe acute rejection episodes in the early weeks after transplantation.

Although rejection of a renal allograft is probably not caused purely by a cellular or a humoral mechanism (with the exception of the hyperacutely rejected kidney), it is easier to discuss the possible roles of both components

of the response to the graft separately. Furthermore, the discussion will be confined in general to vascularized renal allografts in humans as there is not a great deal of information concerning effector mechanisms in other tissue transplants as yet.

HUMORAL MECHANISMS OF REJECTION

Hyperacute Rejection

The best-documented example of antibody-mediated rejection is that of hyperacute rejection. In the classical example, the kidney is rejected within hours of revascularization and rejection is accompanied by a massive infiltration of polymorphonuclear leukocytes and deposition of antibody and complement within the graft (77, 81–84). In the late 1960s the phenomenon of hyperacute rejection was first recognized to occur in the presence of donor-specific leukoagglutinating antibodies (81). Shortly after that, it was found to be a consequence of the presence of lymphocytotoxic antibodies in the recipient which lysed donor lymphocytes in the presence of rabbit complement, a so-called positive crossmatch (77, 82–84). Thereafter it was assumed that all positive crossmatches in a standard lymphocytotoxic assay represented evidence of donor-specific presensitization, and hence a positive crossmatch between donor and recipient became an absolute contraindication to transplantation.

However, in recent years exceptions to the above dogma have been defined, for not all antibodies that give rise to a positive crossmatch are directed against donor histocompatibility antigens of the major histocompatibility complex in man—human leukocyte antigen (HLA) (85). For example, the antibodies reacting with the donor may be autoantibodies or directed at non-HLA targets, and they may be harmless to a subsequent graft (86, 87). Certainly antibodies directed at class-I HLA antigens of the donor will result in immediate rejection; this might be expected, for these antigens are widely expressed in the kidney (88). In the case of class-II antibodies, the situation is not so clear. Certainly class-II antigens are expressed in the human kidney not only on dendritic-like cells, but also on the mesangium, capillary endothelium, proximal tubules, and weakly on the endothelium of large vessels (89, 90). Thus, the presence in the recipient of antibodies specific for donor class-II antigens might be expected to result in antibody-mediated damage to the graft, but the evidence for this remains unclear (85).

Although it is not thought that a cellular response plays a primary role in hyperacute rejection, there are instances where immediate rejection of a kidney occurs in the absence of any demonstrable humoral sensitization in the recipient. The possibility that cells are responsible for hyperacute

rejection here must be considered, bearing in mind the demonstration of a cellular-mediated hyperacute rejection of renal allografts in the absence of antibody in the miniature pig model (91). Furthermore, renal transplantation performed in the presence of a cellular positive crossmatch, where recipient lymphocytes lyse donor lymphocytes in the absence of complement and serum, does increase the likelihood of rejection but does not result in immediate rejection (see below). Thus, this type of rejection is mediated by preexisting antibody specific for donor HLA antigens; the observed cellular changes are merely a response to the initial antibody- and complement-mediated damage.

Accelerated or Acute Rejection

In accelerated or acute rejection, especially where this has proved to be resistant to treatment resulting in loss of the graft, antibody has been considered a major factor in the effector mechanism, mainly because of the histological picture often seen in this type of rejection. Lymphocytotoxic antibodies were first demonstrated many years ago in patients after removal of a rejected renal allograft (92), and these antibodies had donor specificity (93). The failure to detect antibodies in the blood while the graft was in situ was considered to be due to their absorption by the grafted kidney. Furthermore, antibody, both IgG and IgM, were shown to be present in kidneys undergoing acute rejection, but not in grafts with stable function (94).

Histological examination of grafts undergoing accelerated or acute rejection often revealed striking vascular changes such as arteriolar thrombosis, interstitial hemorrhage, and fibrinoid necrosis of the walls of larger arteries (95), all of which were—and indeed still are—considered to be due predominantly to antibody rather than cells. However, although antibodies both donor-specific and nonspecific have been demonstrated by a variety of techniques (96) in the recipient of a renal allograft, and donor-specific antibody has been eluted from rejected kidneys (97, 98), no uniform agreement has developed that the appearance of antibody necessarily precedes acute rejection. Indeed, their appearance may be compatible with apparently stable renal function. Nevertheless, it is difficult not to propose a role for antibody in acute rejection.

Chronic Rejection

Characterized as they are by intimal fibrosis and occlusion of arterioles and arteries, the histological changes of chronic rejection have tended to be attributed to a humoral mechanism. Any firm evidence for this is lacking, however, and these changes probably reflect the aftermath of early acute rejections or a long-standing low-grade rejection.

Another Role for Antibody

A further role could be in an antibody-dependent cellular cytotoxic (ADCC) reaction where the specificity of the reaction is provided by the antibody, but the cytotoxicity is mediated by killer cells that are nonspecific (K cells). Positive ADCC assays after transplantation have been associated both with stable function and rejection (99, 100), and the role of this in vitro phenomenon in vivo remains uncertain.

CELLULAR MECHANISMS OF GRAFT REJECTION

The role of lymphoid system cells in the rejection of human renal allografts has never been questioned, although undoubtedly the modification of the response to the graft by immunosuppression often leads to less evidence of a cellular infiltrate in an allograft undergoing acute rejection than is noted in rejecting skin and vascularized organ allografts in the experimental animal. The relative paucity of the cellular infiltrate in these instances, often associated with marked vascular changes in the graft, as already noted, has led to the belief that this type of rejection is mediated predominantly by antibody rather than cells. Certainly this type of histological picture is seen more often in rejection episodes that do not respond to treatment. However, the possibility that this vascular type of rejection is also mediated by cells cannot be excluded, for undoubtedly the cellular response could be directed against histocompatibility antigens as primary targets on endothelium, with the subsequent vascular changes due to this initial endothelial damage. Some support for this is provided by a detailed electron microscopic study of human skin allografts and autografts in nonimmunosuppressed healthy human volunteers. Widespread microvascular damage was a characteristic early feature of rejection, and this damage was seen initially in those venules, arterioles, and small veins enveloped by lymphocytes (101).

The role of cells in rejection of human renal allografts has depended until recently on morphological observations both of grafts removed after irreversible rejection or at autopsy and of Trucut needle biopsies of the in situ graft. In general a cellular infiltrate, either focal or generalized, was associated with rejection; in the absence of marked vascular changes as described above, this type of rejection could be expected to respond to antirejection treatment. Little information was available on the type of cell making up the infiltrate, but analysis of cells separated from grafts removed for rejection, using assays available at the time, showed that the composition of the infiltrating cell population was made up of T cells, macrophages, and B cells (102, 103). However, the availability of mono-

clonal antibodies that recognize different leukocyte subpopulations has enabled much more precise studies of the nature of this infiltrate in the graft (104–111).

The findings from a number of studies have produced rather conflicting data concerning the nature of the cellular infiltrate during rejection of a renal allograft. In some instances, the infiltrate was said to be composed mainly of T lymphocytes (108, 109), while in others macrophages were the major cell type present (105). However, much of the discrepancy between studies can be attributed to the monoclonal antibodies used and their behavior in frozen and fixed tissue sections; this is discussed below. The studies of the cellular infiltrate in cadaveric renal allografts in the Oxford unit were notable because biopsies were taken before implantation of the graft and at 7, 21, 90, and 365 days after transplantation, as well as at other times for clinical indications (110). In addition, biopsies were taken from patients entered into a controlled trial of cyclosporin versus azathioprine and prednisolone as immunosuppression for renal transplantation. This has provided a wealth of information on the cellular infiltrate in a renal allograft, both in the presence of stable graft function and during rejection, for sequential biopsies were available for analysis in 83 cadaver-graft recipients, approximately half of whom received cyclosporin and half, azathioprine and prednisolone. All of the biopsies underwent a morphometric analysis that allowed a quantitative correlation of the histological findings with the clinical course of the graft.

In brief, as noted by others, a significant leukocyte infiltrate occurred in kidneys undergoing an acute rejection episode or that were removed because of irreversible rejection (110); this infiltrate involved approximately 20% of the area of the histological section. Of note, however, was the significant infiltrate in grafts with stable function during the first 3 weeks after transplantation, when no clinical or biochemical evidence of rejection was present. Furthermore, patients receiving azathioprine and prednisolone tended to have a greater degree of leukocyte infiltration than patients receiving cyclosporin, regardless of whether the graft was undergoing rejection or had stable function (110, 111). Similarly, analysis of the T-cell component of the leukocyte infiltrate—this comprising some 30–35% of the infiltrate—showed the same pattern in that T cells were present in greater numbers in rejection than during stable function, and there were more T cells in grafts when the immunosuppression was mediated by azathioprine and prednisolone, than when it was cyclosporin (Figure 2). Of the remaining components of the infiltrate, monocytes and macrophages comprised about 60%, and NK/K cells less than 10%. Furthermore, there was no alteration in the T4:T8 ratio of the T-cell component in rejecting grafts compared to that in grafts with stable function, and indeed T8 cells

were always present in greater numbers than T4 cells in both situations. Furthermore, the composition of the infiltrate was similar in patients both on azathioprine and prednisolone and on cyclosporin (Figure 3).

Thus, some of these findings are in marked contrast to others. For example, the T lymphocyte has been said by some (108, 109) to be the major cell in the infiltrate, but on the other hand, Hancock et al (105) have reported findings similar to those from Oxford (110, 111)—that T lymphocytes comprise 35% of the infiltrate, with macrophages and NK cells making up most of the remainder. Concerning the T-cell infiltrate, again there is considerable disagreement as to the preponderance of either T4 or T8 cells. Some have found that T8 cells are the major component of the T-cell population (104–106), while others claim that T4 cells are more frequent (106–108). Some of this disagreement may be explained by the monoclonal antibodies used. Some commonly used monoclonal antibodies stain weakly on tissue sections, especially those used for the detection of T4, which in addition show some crossreactivity with macrophages. This has led to incorrect conclusions concerning the T-cell infiltrate. The method of assessment of the infiltrate is another possible source of misinterpretation.

However, if one tries to assess the available data as objectively as possible, then it would seem that the leukocyte infiltrate is a major feature of rejection and is made up of macrophages, NK cells, and T cells—the last

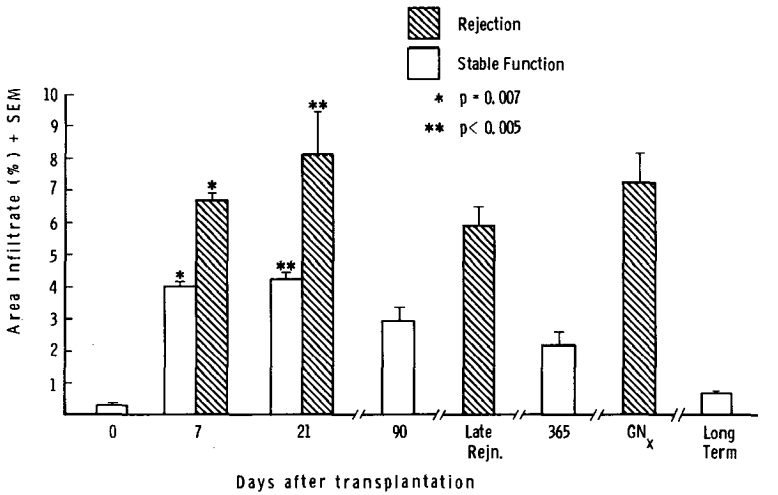

Figure 2 The T lymphocyte infiltrate in grafts undergoing rejection or with stable function at various times after transplantation, including grafts undergoing late rejection or removed for irreversible rejection (GNx). The infiltrate is expressed as a percentage of the total area of the section examined.

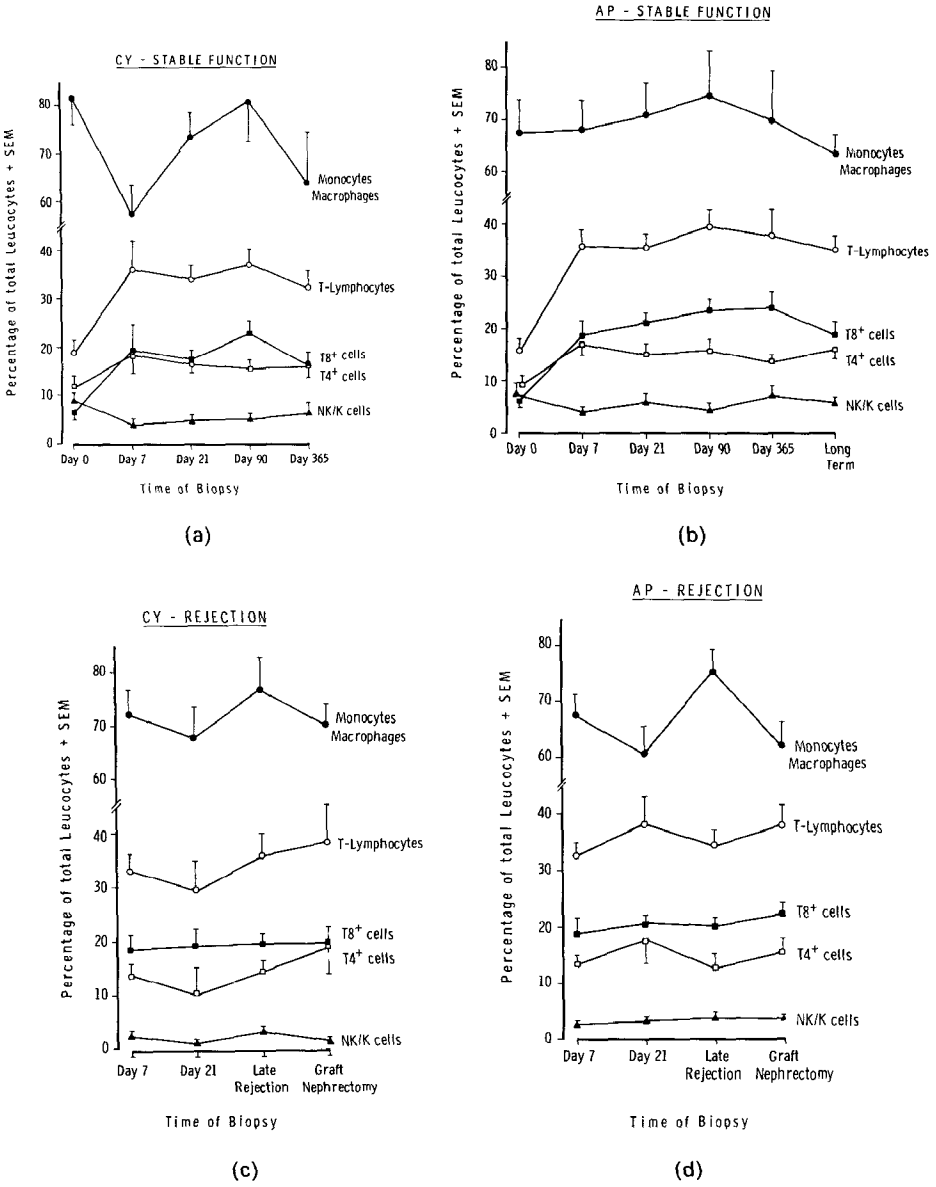

Figure 3 The composition of the leukocyte infiltrate is expressed as a percentage of the total infiltrate at different times after transplantation. (*a*) Patients on cyclosporin with stable graft function; (*b*) patients on azathioprine and prednisolone with stable graft function; (*c*) patients on cyclosporin and undergoing rejection at days 7 or 21 or later after transplantation; (*d*) patients on azathioprine and prednisolone undergoing rejection at days 7 or 21 or later after transplantation.

comprising about 30% of the infiltrate. Of the T cells, T8 cells are present in greater numbers than T4 cells (not a uniform finding), regardless of whether the graft is undergoing rejection or has stable function. Attempts are now being made to isolate these T cells from the graft and to assess the specificity of their cytotoxicity, in bulk culture experiments as well as after the development of T-cell clones from these infiltrates. The development of the technique of fine needle aspiration of a renal allograft, which allows samples to be obtained on a daily basis, may help to determine serial changes in the cellular infiltrate of a rejecting graft and may allow cells to be obtained in sufficient numbers for in vitro studies of function.

Can any conclusions be drawn from these observations as to the nature of the effector mechanism in rejection of a renal allograft in the human? Apart from hyperacute rejection of a graft in a recipient with preexisting antibody against donor histocompatibility antigens, where antibody can be incriminated as the cause of rejection, it is not possible to separate humoral from cellular mechanisms in the modified acute rejection seen in an immunosuppressed recipient, nor in the case of the cellular reaction is it possible to say whether it is a DTH reaction or a classical cytotoxic–T cell response. The T-cell component of the infiltrate comprises both T4 and T8 cells together with a large macrophage component that might be thought to be more compatible with a DTH type of reaction, but the more recent recognition of cytotoxicity mediated by cells of the T4 phenotype and directed at target class-II HLA antigens (112, 113) means that a classical cytotoxic reaction may well be the initial cellular-effector mechanism.

In view of the observations made in a number of experimental models that class-II histocompatibility antigens are expressed in tissues undergoing an inflammatory reaction (discussed in the earlier section), it is interesting to note recent evidence in human renal allografts that class-II antigens are expressed in the kidney during a rejection episode (114). Class-II antigens have a limited expression in the normal human kidney, confined to capillary endothelium, mesangium, dendritic cells, weakly on endothelium of large vessels, and in some kidneys on proximal tubules (90, 115, 116)—although there is some dispute about this latter observation (117–120). The Oxford material has allowed sequential analysis of the expression of class-II antigens to be made during the course of the graft, during both stable function and rejection (121). These sequential biopsies showed not only that class-II antigen was expressed on all tubular cells and more densely on large vessel endothelium during rejection, but that there were fluctuating levels of expression in the same graft. In addition, the expression of class-II antigen correlated with the degree of cellular infiltrate and this in turn with rejection. Furthermore, if the increased class-II expression in the graft regressed, then the graft was more likely to survive.

Thus, in the small series studied, all allografts with normal class-II expression in biopsies taken 90 days after transplantation had survived 2 years, whereas only 60% of grafts with increased expression at 90 days were functioning at 2 yr. Furthermore, the increased expression of class II was much more common in patients receiving azathioprine and prednisolone than in patients receiving cyclosporin, as was the incidence of irreversible rejection. It is possible that some of the immunosuppressive effect of cyclosporin may be related to this decreased expression of class-II antigen in the grafted kidney.

Nevertheless, it is not possible to conclude that the increased expression of class-II antigen leads to rejection, for it could well be merely a marker of the inflammatory reaction associated with rejection. However, it seems not unreasonable to suppose that the appearance of increased amounts of the class-II antigen in the graft might lead to augmentation of the response to the graft, either at the level of induction or more likely at the level of target antigen, particularly that expressed in increased amounts on endothelium.

The comments made hitherto have been confined to changes in the graft itself, but a good deal of work has been directed at both functional and population changes in the blood leukocytes of recipients of renal allografts. For example, donor-specific cellular cytotoxicity has been demonstrated in the blood of patients undergoing severe acute rejection episodes; this cytotoxicity regressed with successful treatment of the rejection episode (96, 99). Again, it has been shown that patients with donor-specific cellular cytotoxicity at the time of transplantation in the absence of a positive antibody crossmatch with the donor do have an increased likelihood of early graft failure from rejection (96, 122). Other assays, such as the mixed lymphocyte culture, cell-mediated lympholysis, leukocyte migration test, and the tanned erythrocyte electrophoretic mobility test, to name but a few, have been used to detect evidence of donor-specific cellular immunity in the host (123). However, the reproducibility of these assays has not been consistent, and although the observations are probably reasonably valid in general, the assays have not become of general use in clinical practice. More recently, monoclonal antibodies have allowed identification of different leukocyte subpopulations in the blood, and although there have been claims that a high ratio of T4 to T8 lymphocytes preceded or was associated with acute rejection (124), these claims have not been substantiated (125, 126); nevertheless, a low T4:T8 ratio does seem to predispose to viral infections (127). Although this approach to the study of effector mechanisms is in its infancy, one cannot help but feel that it will be necessary to continue to study the graft itself, if further light is to be shed on effector mechanisms of graft rejection in the human. Certainly access to the graft in situ is possible—regular Trucut needle biopsies are relatively safe and fine

needle aspirates possible on a daily basis. This allows not only documentation of sequential changes in the graft itself, both during rejection and during stable function, but may also enable functional studies of cells obtained from renal biopsies or aspirates as well. Such observations should help in our further understanding of the effector mechanisms of rejection, when they are interpreted in the light of the observations made in well-defined experimental allograft models.

CONCLUSION

Rejection of a vascularized renal allograft in humans is modified by immunosuppression and is mediated by cells and antibody. There is no question that antibody in a specifically presensitized recipient is responsible for hyperacute rejection of a renal allograft. Although it is more difficult to establish firm evidence for a role of antibody in a modified first-set type of reaction, indirect evidence suggests that antibody does play a part in the effector mechanism. Rejection is invariably accompanied by a leukocyte infiltrate; this is compatible with the importance of the cellular effector response, but not surprisingly it is impossible to say whether this is a DTH-type reaction or a classical cytotoxic–T cell reaction, for the infiltrate is made of macrophages, T cells (both of T4 and T8 phenotype), and NK/K cells. The increased expression of class-II HLA antigen in kidneys after transplantation is associated with rejection and a cellular infiltrate, and this phenomenon may augment the induction of the response or act as a target for the effector arm of the response that might then be mediated by cytotoxic T cells of the T4 phenotype.

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CONTENTS

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STRUCTURAL CORRELATES OF IDIOTOPES

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DIVERSITY OF IMMUNOGLOBULINS AND IDIOTYPY

Immunoglobulin gene expression is unique. Through several mechanisms, an enormous structural diversity of variable-region domains is generated. Variable-region genes are constructed from two (V_L and J_L) or three (V_H, D, and J_H) gene segments that combine without known restriction (1–5). Their combinatorial joining process is variable in that the same two gene segments can be joined in more than one way to generate junctional diversity (6–8). Both mechanisms largely focus diversity on the third hypervariable regions of heavy and light chains. The source of diversity for the first and second hypervariable regions is the polymorphism seen in multiple copies of the V_H and V_L gene segments encoding these regions (9) and in special mechanisms of somatic mutation that seem to produce single-base changes at a rapid rate in rearranged variable-region genes (10–13). The result of these varied mechanisms of genetic diversification is an enormous repertoire of immunoglobulin-binding sites (14).

It was recognized many years ago that the structural diversity of antibody molecules could be detected serologically. Amino acid changes, particularly within the binding site, were expected to generate new antigenic determinants (15). The work of Kunkel et al (16) and Oudin & Michel (17) and their colleagues over 20 years ago confirmed this prediction and established the utility of these determinants, now called idiotypes, in studying variable-region domains. Since then, idiotypic determinants have

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been widely used in the analysis of variable-region inheritance (18–20) and in comparisons of variable-region structure (21). Kunkel and Oudin both showed that antibodies that share antigen-binding specificity often shared idiotypes (16, 17, 22, 23), and this finding has been repeated many times since. Surprisingly, it has been discovered recently that molecules of genetic origin different from that of immunoglobulins, such as T-cell antigen-specific receptors (24–26), hormone receptors (27–30), and virus receptors (31), can share idiotypic determinants with immunoglobulin if they bind the same ligands. This fact has two major implications: There are limited ways of constructing a binding site capable of interacting with a given ligand, and at least some idiotypic determinants reflect the critical binding-site structures. In fact, antibodies to the binding-site idiotopes resemble antigen not only in their affinity for the antigen-reactive molecules of disparate genetic origin, but in their ability to mimic antigen functionally to trigger B and T lymphocytes, in an antigen-specific fashion (25, 32, 33). This antigen mimicry can even extend to a functional substitution for hormones, such as insulin (30) and epinephrine (28), in which antiidiotopes cause responses similar to hormones after binding to the hormone receptors. Thus, idiotopes serve as both structural and functional markers.

In addition, Jerne first recognized that idiotopes could provide means of immune regulation by serving as targets for communication between antigen-reactive cells in the absence of extrinsic antigen (34). An animal is clearly capable of producing antiidiotypic responses to its own immunoglobulins, and antiidiotypic antibodies can either augment (32, 33) or depress (35) the production of idiotype-bearing antibodies. However, it is not yet established that the idiotype network is physiologic, i.e. that normal immune responses depend on idiotype-antiidiotype interactions. Yet, the idea of its central role permeates current immunologic thought and provides an important framework for experimentation.

Finally, observations that immunity can be regulated by passive administration of antiidiotypes led to the possibility of regulating growth of idiotype-bearing lymphoid tumors by antiidiotypic sera (36). Other therapeutic roles for antiidiotype that have begun to be explored include suppression of autoimmune clones, suppression of allograft-responsive clones in transplantation, and stimulation of idiotype-bearing clones as vaccine substitutes.

Although idiotypes are extremely important in a variety of areas of immunology, our knowledge about the structure of idiotypic determinants is fragmentary. To assign structure to particular idiotopes has been difficult. In part, this is because the extraordinary diversity of immunoglobulin structure often leads to multiple structural differences between idiotope(+) and idiotope(−) molecules. Therefore, idiotopes have been assigned

successfully when large panels of immunoglobulin molecules were analyzed structurally to localize more precisely the relevant variables.

The second major difficulty in molecular assignment of idiotopes has to do with the nature of antigenic determinants themselves. Ideas about what constitutes an epitope range from those that see a protein as expressing an unlimited continuum of surface structures, each potentially an epitope (37), to those that envisage epitopes to be portions of molecules with intrinsic attributes that make them antigenic (38, 39). Those who hold to the former view could justifiably question the validity of molecular assignment of a particular idiotope because no general principles about idiotope structure would emerge. On the other hand, if only certain parts of variable domains are intrinsically antigenic, the search for molecular correlates of idiotypy will be both important and fruitful.

The purpose of this review is to summarize current information about the molecular basis of idiotypy and to focus attention on strategies that may be useful in idiotypic definition. For more detailed discussion of the biological aspects of idiotypy and other perspectives on their structure, several excellent reviews can be consulted (40–43).

NATURE OF IDIOTYPES

Before considering the molecular basis of idiotypy, it is important to discuss first the nature of idiotypes, their definition, detection, general location, and some general principles of serology.

It is very easy to detect idiotypes; it is another thing, however, to define them. Typically, an antiidiotypic serum is what remains after extensive absorption of a heterologous antiserum raised against a homogeneous immunoglobulin. The absorbent essentially determines the mix of anti-idiotopes remaining, and the cynic might claim that no two antiidiotypic antisera are alike because normal serum immunoglobulin, the traditional absorbent, is undefinable and probably irreproducible. However, careful choice of immunoglobulins for absorbents generally provides a more specific, definable reagent. Certainly, the generation of a monospecific reagent would require absorption with immunoglobulins that share all determinants (for both constant and variable domains) except the desired one. Alternatively, only a single epitope would be detected by a multi-specific reagent, if an immunoglobulin sharing only the single epitope with the immunogen were available to serve as the ligand. In practice, the number of epitopes detected by a given antiidiotypic serum is nearly always unknown or at best only approximated.

Monoclonal reagents have solved many of these uncertainties and have ensured that different investigators now can use standard reagents that

react with a single epitope. Determination of the location of that epitope, the topic of this review, involves several steps. First, the distribution of the epitope among other antibodies with similar or different specificities provides clues to its possible location. An epitope found exclusively on the immunogen might be determined by a unique somatic mutation; an epitope found on some or all of the antibodies, however, could be a reflection of V_H, D, or V_L encoded structures, either alone or in aggregate. Secondly, reaction with separated H and L chains, or homologous and heterologous H-L recombinants, will permit localization of some determinants.

Another revealing serologic parameter only infrequently used in idiotypic analysis pertains to the variability of the epitope among different immunoglobulins. Figure 1 shows the pattern of expression of two idiotopes among a panel of murine monoclonal antistreptococcal group A carbohydrate (GAC) antibodies (44). The height of each line represents the extent of expression of each idiotope on anti-GAC antibodies, based on the binding of monoclonal antiidiotopes. It is clear that proteins are either equally IdX(+) or IdX(−), but that IdI-3a is expressed to a variable degree on the majority of anti-GAC antibodies. One can infer from this that IdX resides in a germline-encoded portion of the variable domains subject to only modest variability and that IdI-3a is in a highly variable region, most likely involving the binding site. This was supported by the fact that IdI-3a was no longer accessible to antiidiotope when the hapten, N-acetyl glucosamine, occupied the binding site, whereas IdX was not affected (45). Clearly, hypervariable regions, particularly HV3, are the most likely locations for epitopes expressing a continuum of variants, like IdI-3a.

It is also clear that the kind of assay used to measure idiotope expression is important. Direct binding assays, like those depicted in Figure 1, are able

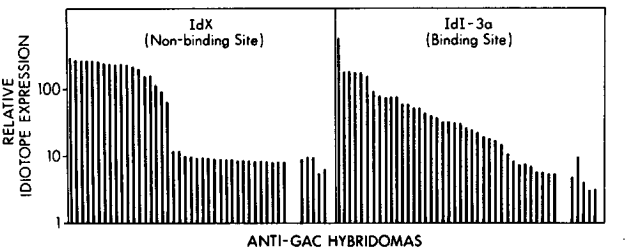

Figure 1 Idiotopes vary in their polymorphism. Two monoclonal antiidiotopes, detecting a nonbinding site determinant (IdX, left) and a binding site determinant (Id-3a, right) on anti-GAC antibodies of A/J mice, were tested on a panel of monoclonal anti-GAC antibodies. The height of each line represents the degree of reactivity of antiidiotope with a separate monoclonal anti-GAC antibody. The values are relative; 10 or below represents background. (Modified from 44.)

to detect low-affinity cross-reactions. In competitive binding assays where one measures the relative capacities of a panel of antibodies to block binding of a labelled idiotope-bearing ligand to antiidiotope, the relative affinities of inhibitor and ligand for the antiidiotope are important. Low-affinity binders may not be able to inhibit, at a reasonable concentration, the binding of high-affinity ligands, whereas the reverse is not true. Thus, results that emerge from competitive binding assays are particularly sensitive to the relative affinities of ligands and inhibitors for the anti-idiotope. In addition to determining whether idiotope expression is modified by hapten or haptens coupled to carriers of a larger molecular weight, the relative positions of idiotopes on the surface of variable domains can be determined by cross-inhibition studies with other antiidiotopes and anticonstant-region reagents (45). Armed with appropriately targeted reagents, it should be possible to develop a topographic map of idiotopic determinants. Finally, in some instances it has proved fruitful to modify immunoglobulins chemically to determine whether particular idiotopes depend on modified residues or not (46, 47). Thus, a great deal can be learned about the characteristics of idiotopes by serologic means alone.

A variety of descriptive terms have been applied to idiotopes, public vs private, cross-reactive (CRI) vs individual, IdX vs IdI, that essentially reflect the relative degree of expression of a determinant among antibodies of the same specificity. As will be seen, none of these operational definitions has been put on satisfactory structural grounds yet. Separation of idiotopes into binding site and nonbinding site determinants is informative and probably important, but again structural explanation of this is lacking. It is therefore probably still premature to consider categorization of idiotopes other than by the operational terms currently in use.

STRUCTURAL CORRELATES OF IDIOTOPES

Although many families of antibodies have been studied idiotypically, knowledge of only a few systems has progressed to a point where structural correlations of idiotopes can be considered. Several of these systems are described here.

Idiotopes of Antidextran Antibodies

The immune response in mice to B1355S dextran is very simple. Only BALB/c or other mice with the Igh-C^a haplotype produce significant levels of antibodies to dextran (48); IgM, IgG3 (49), and IgA all paired with λ_1 L chains are the dominant isotypes. Essentially all of the antibodies are $\alpha(1 \rightarrow 3)$ $\alpha(1 \rightarrow 6)$–specific, but they are heterogeneous with regard to fine specificity (50). At least half of the antidextran antibodies share an idiotypic

determinant termed IdX with two BALB/c myeloma proteins, M104 and J558 (51, 52). In addition, M104 and J558 possess unique determinants termed IdI(M104) and IdI(J558) that are shared by much smaller fractions of the total antidextran antibody population.

Amino acid sequence analysis of antidextran hybridoma proteins has been most rewarding in identifying the residues critical to the expression of IdI(J558), IdI(M104), and IdX idiotopes (Figure 2). Because nearly all antidextran monoclonal antibodies studied to date bear $\lambda 1$ light chains, which are known to arise from single V and J gene segments, structural analysis has focused on V_H sequences. Of the antidextran immunoglobulins 20 have had complete V_H regions sequenced (53); 15 of these are shown here. Interestingly, all of these sequences are unique, yet most vary from J558 and M104 by only a few residues. The proteins are most likely created by the combinatorial joining of 2 or more related V_H segments, an unknown number of D segments generating 10 different sequences at H100 and H101, and 4 J_H segments. Most striking are the sequences of M104 and J558 that are identical in the V_H and J_H segments and differ only in the two-residue-D segment. Since L chains of these proteins are identical (54), the expression of

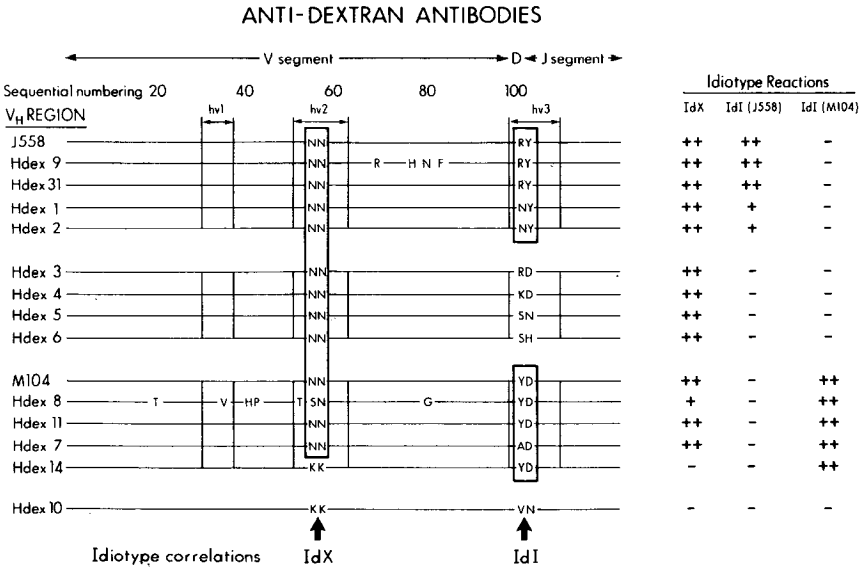

Figure 2 Structural correlates of idiotopes on antidextran antibodies. Amino acid sequences of V_H regions of mouse monoclonal antidextran antibodies are shown. All antibodies have λ_1 L chains. Lines indicate identity to the sequence of the first protein; letters (Dayhoff code) show differences or regions correlated with idiotopes (boxed area marked by arrows). Idiotype reactions of each protein with several heterologous antiidiotopes is shown on the right. (Modified from 53, 56.)

the IdI(M104) or IdI(J558) idiotypes must be dependent on the D segments. Computer modeling of J558 and M104 (Figure 3) suggests that the D segments are prominently exposed within the binding pocket and may lead to significant structural changes that could account for the idiotypic determinants themselves. (See 55 for a discussion of computer modelling of antibodies.)

The correlation of D segments with IdI(M104) or IdI(J558) idiotype expression is striking (56; Figure 2). All three proteins with RY residues (Dayhoff code) in their D segment express the IdI(J558) idiotype. Partial expression of IdI(J558) is seen in proteins with NY D segments. Further, IdI(M104) expression is correlated with YD and AD in the D segment. However, not all IdI(+) proteins have related D segments. Recently, Kearney and colleagues generated several monoclonal antiidiotype antibodies that distinguish M104 and J558, thereby qualifying as IdI-specific reagents (57, 58). Although some of these monoclonals recognize determinants similar to IdI(M104) and IdI(J558), others do not. Thus, it is possible that proteins differing only by two amino acids may bear several idiotypic differences.

The IdX idiotope appears to depend on two amino acids in HV2 (56). Proteins differing only in residues H54 and H55 are IdX(+) and IdX(−).

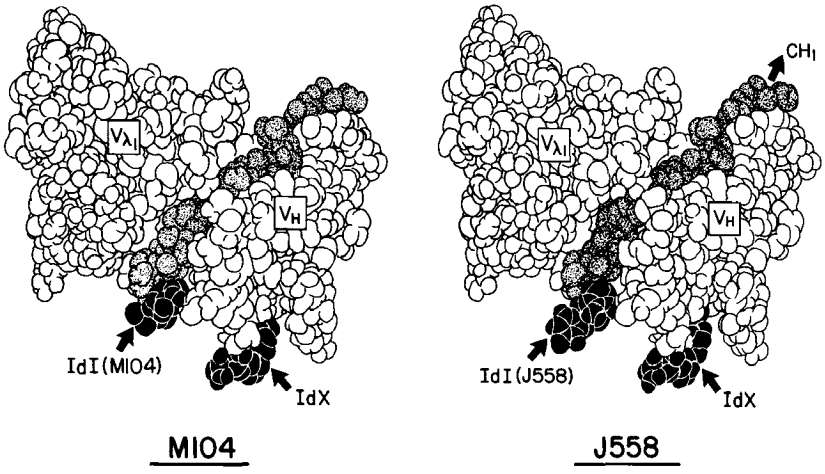

Figure 3 Computer-generated models of variable domains of two antidextran antibodies, M104 and J558, showing approximate positions of amino acids correlated with three idiotopes, IdX, IdI(M104) and IdI(J558). These two proteins differ by only two amino acids encoded by D that correlate with IdI(M104) and IdI(J558). The binding sites face to the lower left; IdX is on the lip of the binding site and the IdI determinants emerge from the floor. The J_H region (grey) lies in a groove between V_λ (upper left) and V_H (lower right) and connects with CH_1 (top right). (Models courtesy of Richard Feldmann and Michael Potter, NIH.)

All proteins that are IdX(+) have NN at these positions. Thus, although some questions still remain concerning IdX and IdI expression, available sequence information strongly suggests D segment involvement for IdI expression, and residues H54 and H55 in IdX expression.

From these studies, it is clear that a few amino acids are strongly correlated with the expression of particular idiotypic determinants. However, from this information alone it is impossible to determine whether the critical amino acids are themselves the idiotypic determinants or whether they are only secondarily involved in forming the idiotopes, possibly by conformational influences on other parts of the V domains.

Idiotopes of Anti-PC Antibodies

Most mouse immunoglobulins generated to phosphocholine (PC) bear the dominant T15 idiotype, with much smaller portions of the response bearing the M511 or M603 idiotype. In BALB/c mice, the prototype strain, anti-T15 serum can inhibit 70–80% of the PC-specific plaque-forming cells (59–61); in addition, the majority of monoclonal anti-PC antibodies express the T15 idiotype (10, 62).

Molecular study of PC-specific immunoglobulins has indicated that the entire response is generated from a single germline V_H segment gene (63) in combination with one of three V_K light chains (64–67). Each of the three major idiotypic families is correlated with the expression of a single light chain (62). Hence, T15(+) molecules are composed of a heavy-chain-gene segment encoded by the VH-PC paired with the VK22 light chain, while M511(+) molecules use the same heavy chain paired with the VK24 light chain. The reciprocal, however, is not true, since some VH-PC-VK22 molecules are T15 idiotype(−). In addition to the limited V_H and V_K repertoire, the anti-PC response is also restricted to the JH_1 and JK_5 joining segments; however, extensive diversity in the D segment suggests that at least three D genes are used (68).

Somatic mutations have been demonstrated in both the heavy and light chains (69, 70). Differences between the amino acid sequence predicted from the single germline V_H gene segment and the actual PC-specific heavy-chain protein are due to somatic mutations. Of over 40 published sequences, at least 24 have V_H segments identical to the germline sequence (10, 65, 68), with the remainder demonstrating somatic mutation leading to at least one amino acid substitution. Even the most divergent PC-specific protein, however, still shares 88/95 residues with the germline T15 V_H segment. In comparison, M104, a dextran-specific immunoglobulin, shares only 45/95 residues with T15 V_H segment.

Finally, N-junctional diversity and gene conversion also play a role in the anti-PC repertoire. N-junctional diversity has been postulated to account

for nucleotides present at the VDJ gene junctions that could not have been contributed by V_H, D, or J_H gene segments (68). Gene conversion has been implicated in a single anti-PC clone, which appears to have accepted portions of two related V_H genes to encode its V_H region (71).

Several attempts have been made to characterize the T15 idiotype. In particular, chain recombination experiments using sequenced H and L chains have suggested that the idiotype is complex with direct or indirect contributions made by the L chain and the D segment (72, 73). Recent study of monoclonal antiidiotope antibody (anti-C3-16 Id) showed patterns of reactivity with anti-PC antibodies consistent with involvement of both D-segment and light-chain determinants in expression of the idiotope (74; Figure 4). Only antibodies with VK22 light chains and the dominant D region YYGSS and YYGNS are reactive with anti-C3-16 Id (solid box, Figure 4). Further, certain Id-C3-16(−) anti-PC antibodies, not shown in this figure, are identical to Id(+) proteins in V_H region but are paired with VK8 light chains instead of VK22. Heavy- and light-chain mixed molecules from idiotope(+) and (−) antibodies supported this conclusion. The T15 IdX determinant, measured by A/He anti-T15 antisera, also has been localized tentatively to this region (Figure 4; solid and hatched box), although, in view of the multiplicity of determinants detected by allo- and xenoanti-T15 sera, it is likely that other regions of T15 will be identified as idiotopes in the future.

Furthermore, the reaction between anti-C3-16 Id and its idiotope is

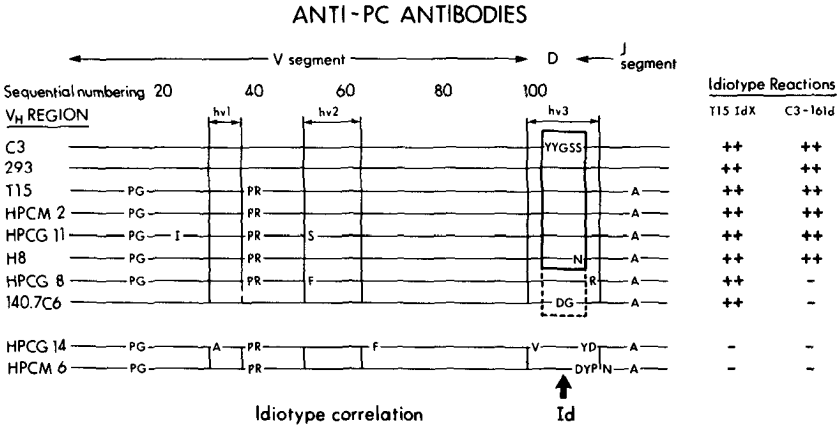

Figure 4 Structural correlates of idiotopes on anti-PC antibodies. V_H regions of anti-PC antibodies (all expressing VK22) from several different strains of mice were sequenced. Reactivity with A/He anti-T15 and a monoclonal antiidiotope is indicated on the right. The region of structural correlation with idiotope expression is indicated by the boxed areas and the arrow (T15 IdX, solid + hatched box; C3-16Id, solid box). (Data from 74.)

blocked by the hapten PC; this is interesting because the only contact residues for PC in M603 (an anti-PC antibody that expresses VK8 and a similar V_H sequence to the C3-16 Id(+) molecules) are found in HV1 and HV2. Nonetheless, it is interesting to consider an idiotope that is dependent on both D region and V_K elements. It is possible that the actual determinant is found on one partner chain, either H or L, but conformational influences from the other permits expression of the idiotope. Along these lines, Fulton et al recently reported an idiotope expressed on K chains from anti-GAC antibodies that was dependent for expression on the H partner (75). K chains that expressed idiotope in the absence of H chains actually lost the idiotope when paired with inappropriate H chains. For anti-PC antibodies, the other possibility is that both D and VK22 form the C3-16 Id. The D region comes in close proximity to HV1 of the L chain, an area of considerable variability among the three V_K genes used in anti-PC responses. Distinguishing between these several explanations for the location of the idiotope will likely require new approaches.

Idiotopes of Antigalactan Antibodies

Anti-$\beta(1 \rightarrow 6)$-D-galactan antibodies in mice have also been studied structurally and idiotypically. A series of myeloma proteins (76) and hybridomas (77) from animals immunized with galactan-containing antigen have been sequenced. The majority of these immunoglobulins have K chains with identical V_K segments coupled with different J_K segments. However, the V_H domains show considerable diversity resulting from the pairing of one or a few V_H gene segments, an uncertain number of D, and at least three J_H genes combined with extensive diversity at the V_H-D and D-J_H junctions and with probable somatic mutations. Allogeneic antiidiotypic sera detect a variety of idiotopes on this family of proteins, two of which are strongly correlated with HV3 amino acid sequences (Figure 5). The Id HG3-bearing group have an invariant GYYGY sequence in HV3 absent from idiotype(−) molecules, although some (−) antibodies differ by only a single amino acid from this sequence. Similarly, the Id HG6 group uniquely has LGHYGY in the D region. Heavy- and light-chain recombination experiments support the heavy-chain location of these idiotopes, although contributions of light chains to these determinants are not ruled out (78).

Idiotopes of Anti-NIP Antibodies

Rajewsky and his colleagues have applied an important new approach to the localization of idiotopes by selecting from idiotope(+) cell lines spontaneous mutants that have lost the idiotope. These investigators have described a variety of idiotopes on mouse antibodies to the hapten 4-hydroxy-3-nitro-5-iodophenylacetyl (NIP), including Ac38, a determinant

outside the binding site, and Ac146, a determinant inside the binding site (79). Variants were isolated from a NIP-specific cell line B1-8.δ1 expressing both determinants. One mutant that lost the binding-site determinant also lost the ability to bind NIP (80). This variant, B1-8.V1, differs from the parent by 10 amino acids in the V_H domain, resulting from recombination of the rearranged VDJ gene of the parent and a similar, presumably unrearranged, V_H gene (Figure 6). It is possible that Ac146 expression correlates with HV2 structures. An even more interesting variant, B1-8.V3, lost the nonbinding site determinant, Ac38, retaining both the binding-site idiotope and NIP specificity (81). This protein differs from the parent by a single amino acid in HV3. Thus, various idiotopes on NIP-specific antibodies involve, at a minimum, amino acids in HV2 and HV3, as well as pairing with appropriate L chains.

The approach of isolating idiotope-loss mutants provides an opportunity for determining each of the regions of an immunoglobulin critical for the expression of a given determinant. With sufficient effort, researchers should be able to map and precisely delimit even the most complex determinant.

Idiotopes of Anti-ARS Antibodies

Another extensively studied family of antibodies involves those specific for *p*-azophenylarsonate (ARS). Essentially, three families of anti-ARS antibodies, ARS-A, -B, and -C, are generated from very few germline genes and

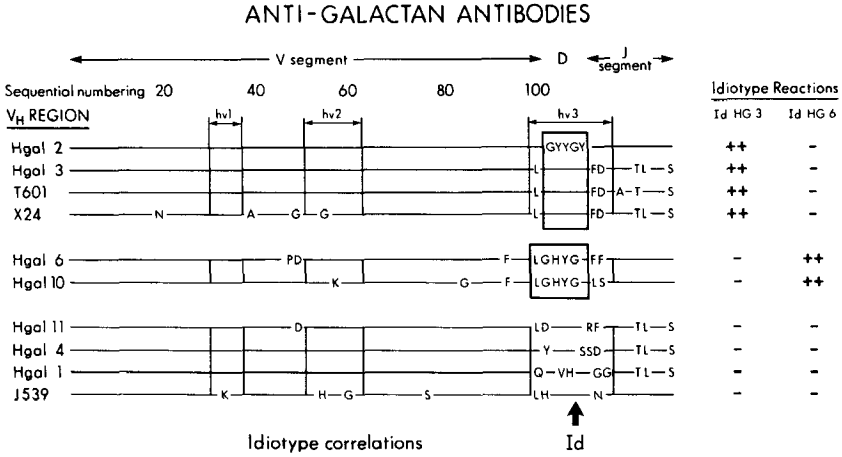

Figure 5 Structural correlates of idiotopes on antigalactan antibodies. V_H regions of several BALB/c myeloma and hybridoma-derived antigalactan antibodies are shown along with reactivity patterns with two allogeneic antiidiotopes. All antibodies use very similar L chains. Correlates of idiotopes are indicated by boxes and an arrow. (Data from 77.)

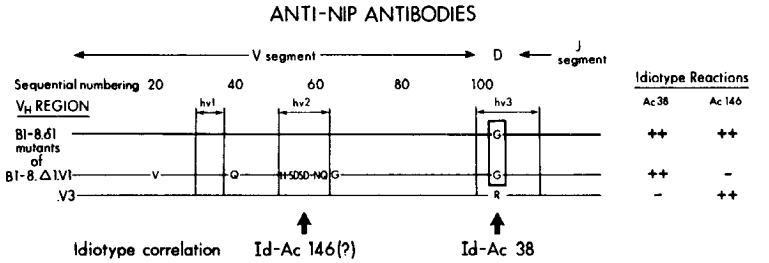

Figure 6 Idiotope-loss variants of anti-NIP antibodies. Variants B1-8.δ.V1 and B1-8.δ.V3 were isolated from NIP-specific hybridoma cell line B1-8.δ1 on the basis of selective loss of reactivity with one of two monoclonal antiidiotopes, Ac38 and Ac146. Amino acid sequences of V_H regions of the parent and variant-derived antibodies are shown. Idiotope expression and sites of structural correlation are shown. (Data from 80, 81.)

can be detected by heterologous antiidiotypic sera reactive with V_H-associated determinants (20, 21, 82). The three families are expressed to different degrees among various strains of mice. The dominant component of A/J responses to ARS-KLH is ARS-A (21), whereas this family is virtually absent from that of BALB/c mice. ARS-A is nearly always generated from single V_H segment, D segment, and J_H segment genes. The absence of this V_H segment gene from the BALB/c genome seemed to explain the absence of the V_H-associated idiotype, CRI_A, from BALB/c anti-ARS sera (83, 84). However, Urbain and colleagues demonstrated that treatment of BALB/c mice with anti-CRI_A and ARS-KLH elicited $CRI_A(+)$ anti-ARS antibodies (85, 86). Upon sequencing the V_H domains of two of these unusual antibodies, Meek et al found them to be over 45% different from A/J $CRI_A(+)$ antibodies (87; Figure 7). However, once again the idiotope is strongly associated with sequences in the D region. Surprisingly, BALB/c CRI_A-associated D regions were paired with two different J_H gene segments, and

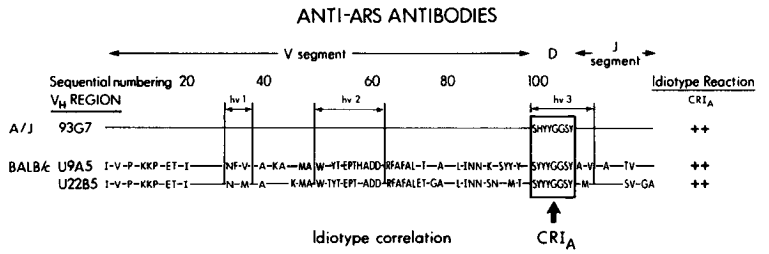

Figure 7 Structural correlate of an idiotope on anti-ARS antibodies. Hybridoma producing $CRI_A(+)$ anti-ARS antibodies were selected from two strains of mice, A/J and BALB/c, with disparate V_H genomes. Amino acid sequences of V_H regions of three $CRI_A(+)$ antibodies are shown and the most striking structural correlates of CRI_A are indicated. (Data from 87.)

neither of them corresponds to the J_H segment that typically appears in A/J $CRI_A(+)$ molecules. In addition, the BALB/c V_H gene segment could hardly be more different from the A/J V gene segment. Further, A/J $CRI_A(-)$ antibodies were shown by Gridley et al to express the same V_H gene segment with different D segments (88). Not only does this result serve as a striking illustration of the importance of D regions to idiotypy, it speaks to its role in binding specificity.

At the same time, however, the two BALB/c $CRI_A(+)$ antibodies expressed L chains that in H-L recombination experiments were required for CRI_A expression. Therefore, it is still not clear whether CRI_A resides on D, L, or both.

USE OF PEPTIDES IN LOCALIZING IDIOTOPES

In all of the examples given so far, with the exception of the V_K idiotopes on anti-GAC antibodies, idiotope expression depends on specific H-L pairs. And, as we have just seen, distinguishing which chain of the pair bears the determinant or deciding whether the idiotope spans both chains is difficult. Probably most idiotopes require specific H-L pairs (89, 90), although exceptions are not uncommon (75, 91, 92).

Idiotypes have been defined until recently using antisera or monoclonal reagents derived from animals immunized with intact immunoglobulins. Clearly, this permits detection of complex, conformation-dependent idiotopes that are difficult to characterize. The last few years have seen an increased interest in using synthetic peptides or peptide fragments of proteins as immunogens (93). As far as idiotopes are concerned, a major advantage to this approach is that the determinant location is predetermined. Several groups of investigators have recently explored the usefulness of peptides in the study of idiotypy. Chen and colleagues have been successful in generating antiidiotypic reagents using synthetic peptides from HV2 and HV3 of human rheumatoid factor (94, 95). Anti-HV3 seemed to define a private idiotype binding only the protein with the identical sequence, while antisera to the HV2 region defined a cross-reactive idiotype present on many rheumatoid factors, including some that were not sequence identical. Similarly, McMillan and colleagues found HV3 peptides of antidextran antibodies (Figure 2) to be effective stimulators of antiidiotypic antibodies (96). In many cases, unabsorbed rabbit anti-HV3 could distinguish between two immunoglobulins (M104 and J558) whose variable region domains differed by only two amino acids (Figures 2 and 3) (M. V. Seiden, R. Heuckeroth, B. Clevinger, S. McMillan, R. Lerner, J. M. Davie, submitted for publication). In addition to confirming that the IdI(M104) and IdI(J558) determinants are located in

HV3, these results illustrate the fact that HV3 idiotopes do not always require L-chain influences. Interestingly, antisera to HV1 and HV2 peptides of antidextran antibodies were less satisfactory as immunogens, in spite of the presumed location of IdX in HV2. Similarly, HV1 and HV2 peptides were less effective stimulants of antiidiotope responses in two other recent studies (97, 98) in which HV3 peptides were highly immunogenic. Finally, new idiotypic determinants were discovered by antisera raised to JH_1 peptide (99). It seems, in this case at least, that a single peptide is capable of eliciting multiple binding site–associated antiidiotopes.

It is intriguing, from several perspectives, that the third hypervariable region of H chains emerges as an important region for idiotypy. In all of the systems discussed in this review, HV3 and, to a lesser extent, HV2 are implicated as sites for idiotypic determinants. Because this region lies at the β bend between the eighth and ninth β-pleated sheets, it is tempting to speculate that this region may have high mobility and therefore increased immunogenicity, at least according to some (38, 39). The role of L chain may be to provide proper positioning of this H-chain region. Clearly, the hypervariable regions for both V_H and V_L approximate one another (100), and interactions are likely.

Although HV3 has a clear role in idiotope structure, it should not be concluded that other parts of variable domains are not involved in idiotypy; rather, there is considerable evidence that many other sites are involved, and future structural studies will likely locate them. At the least, however, the present state of knowledge of idiotope structure suggests that general principles may emerge from this line of investigation that may permit predictions of idiotope location and structure. In time, with better understanding of the role of idiotypes in immune regulation and immunotherapy, it may be possible to bring practical application to this knowledge.

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CONTENTS

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REGULATION OF B-CELL DIFFERENTIATION:

Interactions of Factors and Corresponding Receptors

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INTRODUCTION

The antibody response to nominal antigen such as hapten-carrier conjugates involves the cooperation of hapten-specific B cells and carrier-specific helper T cells (1–4). In addition to the carrier specificity, helper T cells recognize determinants encoded by genes in the class-II major histocompatibility complex (MHC) on antigen-presenting cells as well as on B cells (5–13). The interaction between helper T cells and antigen-presenting accessory cells (APC) is genetically restricted by products of the major histocompatibility complex (14–16). However, the cellular interactions between helper T cells and B cells have been reported as restricted by self-MHC products (5–7, 10–12, 17) or not restricted by the MHC elements (18, 19).

Since genetic restrictions imposed by products of the MHC are considered as a requirement for the recognition by T cells of self-MHC determinants expressed by non-T cells (APC), this controversy can be explained on the basis of whether T cells must necessarily recognize the MHC determinants expressed by B cells in order to trigger them to secrete antibodies. Since the original description of a role for T cells in the antigen-dependent stimulation of B cells, a great deal of effort has gone toward elucidating the mechanism by which helper T cells function.

A series of studies demonstrated that some members of the B-cell population participate in cognate cellular interactions with helper T cells. Cognate interaction refers to the recognition of both antigen and a class-II MHC molecule on the B-cell surface as a requirement for activation. Other

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B-cell populations have the property to be stimulated by several unrestricted nonspecific lymphokines. Attempts to isolate and characterize soluble helper-T-cell factors and to determine how these act in synergy with antigens to stimulate B cells have not yet established their number, their function, or whether these factors act in a definite order. Recent studies suggest that induction of B-cell proliferation requires T-cell factors that are distinct from those that induce B-cell differentiation to antibody-secreting cells (20–22). In a murine system with the use of antiimmunoglobulin (anti-Ig) as a polyclonal B-cell activator, Howard, Paul, and coworkers (20, 21) suggested that a series of exogenous signals were necessary for anti-Ig-stimulated B cells to proliferate and differentiate. Thus, anti-Ig was found to stimulate resting B cells to enter an early G1 phase, where they acquired responsiveness to a T cell–derived factor, B-cell growth factor (BCGF). BCGF was found to be necessary to drive the cells into late G1 phase. At this stage of action, they required a macrophage factor, interleukin 1 (IL-1), to enter the S phase (23, 24). IL-1 and/or BCGF-driven B-cell proliferation is thought to generate daughter cells capable of differentiating into Ig-secreting cells as a consequence of responses to a battery of B-cell differentiation factors (BCDF). The sequence of B-cell differentiation events remains obscure, however.

In this review we consider the current understanding of B-cell differentiation factors that govern late events in the development of antibody responses. Although it has recently been reported that B cells also produce BCGF (25) and BCDF (26), and some B-cell populations are regulated in their proliferation and differentiation by macrophage-derived IL-1 (20, 21, 25) and accessory cell–derived factor(s) (27), we concentrate the discussion on T cell–derived B-cell differentiation factors. After an initial listing of the distinct T cell–derived B-cell differentiation factors identified to date, an attempt is made to illustrate the interactions of involved factors and corresponding receptors. We refer to this background information when analyzing the data to demonstrate how these T cell–derived B-cell differentiation factors play an important role in antibody responses under physiological and some pathological conditions. We then attempt to draw some general hypothesis about the mode of action of B-cell differentiation factors that are influenced by class-II MHC-mediated cell-cell interactions.

FACTORS INVOLVED IN THE DIFFERENTIATION OF ACTIVATED B CELLS

After the original discovery of T-B cell collaboration in the antibody response, several investigators further recognized that the B-cell response

could be triggered under certain circumstances in which B and T cells were independently stimulated by determinants present on two distinctly separate molecules. The mechanism underlying such B-cell triggering was interpreted to be the mediation of the T-cell effect via the release of molecules with T cell–replacing factor (TRF) activities (28–33).

In fact, several laboratories have demonstrated that resting B lymphocytes can be stimulated to proliferate and differentiate into immunoglobulin (Ig)-synthesizing cells by anti-IgM antibodies together with a series of T cell–derived growth and differentiation factors (34–39). Howard, Paul & associates, for example, have utilized the anti-IgM polyclonal B-cell activation system as an effective means of separating proliferation and differentiation events. The proliferation of anti-IgM-activated B cells can be stimulated by B-cell stimulatory factor p1 (BSFp1) (34, 39). B-cell growth factor-I (BCGF-I) or B cell–stimulating factor, provisional 1 (BSFp1) has been defined as a T cell–derived lymphokine that acts as a costimulator of polyclonal B-cell growth in B cells cultured with anti-μ, anti-δ, or anti-Ig. Based on a number of studies they suggested that anti-Ig induces cell enlargement, entry into the G1 phase of the cell cycle, and expression of receptors for BSFp1. BSFp1 subsequently induces entry of the cells into S phase. However, this process is unable to induce production of antibody response. Recently, by adding BSFp1 prior to anti-Ig, Vitetta and associates (40), and Paul and colleagues (137), found evidence that BSFp1 renders cells susceptible to anti-Ig-mediated entry of cells into G2/S phase. In contrast, if cells were first treated with anti-Ig, washed, and then cultured with BSFp1, they did not enter S phase. These results suggest that BSFp1 acts on the resting B cells not as a growth factor but rather as a lymphokine that prepares B cells for anti-Ig-mediated activation.

Moreover, Nakanishi et al (41) reported that differentiation to high rate Ig synthesis of such activated B cells depends upon two additional T cell–derived factors. One (B151-TRF), required early in the course of the response, is found in the supernatant of the B151K12 T-cell hybridoma line established in our laboratory (42). The second factor, (EL-TRF), can be added relatively late in the course of the culture (43) and is present in supernatants of EL-4 thymoma cells that have been stimulated with phorbol esters. These two factors are further distinguished by kinetic studies that clearly demonstrated their independent roles in the development of Ig-secreting cells: B151-TRF is required in the last 48 hr in a 4-day culture, whereas EL-TRF appears to function in the final 24 hr. The actions of B151-TRF and EL-TRF are apparently antigen nonspecific and are dependent on the presence or prior action of BSFp1. EL-TRF is not active in the anti-IgM synergy assay described above by Howard & co-workers

(20, 21). It is, therefore, readily distinguished from the proliferation cofactor BSFp1.

Data of Nakanishi et al (44), moreover, demonstrated that a combination of lymphokines was required for a maximum IgM response in their system and that if any one of these activities was missing, there was marginal or no IgM response from resting B cells. Thus, this system allowed a detailed study of the relationship between each step in the B-cell response process and the appearance of mRNA involved in the secretion of IgM. These investigators examined levels of membrane-type IgM heavy-chain (μm) mRNA, secretory-type IgM heavy-chain (μs) mRNA, and total μH-chain mRNA, as well as J-chain mRNA and κ chain mRNA in resting B cells and in anti-IgM-stimulated B cells that had been cultured with various T cell–derived growth and differentiation factors. Their results indicate a striking induction of μs mRNA and J-chain mRNA, and an increase in the μs/μm mRNA ratio which occurred at the end of the response process, when all T cell–derived growth and differentiation factors have acted upon these cells. No major specific changes were detected in cells that had completed only a part of the total response. In their system, 10–30% of the cells cultured with anti-IgM in conjunction with these three sets of T cell–derived factors possessed cytoplasmic IgM.

In these and other experiments (45), the presence of B151-TRF was obligatory for the effective induction of antibody-forming cells. B151-TRF was originally defined in antigen-specific responses as a molecule that induces antigen-primed B cells to undergo specific antibody-forming cell development in the presence of antigen (46). The T cell–replacing nature of the B151-TRF was determined on the basis of its triggering of the antihapten IgG PFC responses of T cell–depleted hapten-primed spleen cells upon stimulation with the hapten-heterologous carrier conjugate. More definitively, DNP-D-GL (D-glutamine: D-lysine copolymer), a compound to which the T cells do not respond, was also able to trigger DNP-primed B cells in the presence of the B151-TRF (42). Leibson et al (47, 48) have reported that interferon (IFN) could induce terminal differentiation of B cells in combination with IL-2. Our results, however, distinguished B151-TRF from a number of previously described lymphokines, because B151-TRF material did not show any significant BSFp1, IL-2, or IFN activity. Thus, the B151-TRF that is biologically distinct from IL-2 and IFN can directly induce terminal differentiation of a portion of antigen-specific B cells to become antibody-forming cells.

In view of the differences in assays utilized in the above two laboratories, it cannot yet be definitely stated that the TRF activity found in culture fluid supernatant (CFS) of B151 T-cell hybridoma used in our assay is identical to the activity demonstrated by Nakanishi et al (43, 44). Nevertheless, the

similar biological role and restricted cellular source of the activities studied in both laboratories as well as the fact that B151-TRF is active in the dextran sulfate synergy assay may indicate that the same molecule may be responsible for the functional activity observed in both assays.

B-CELL DIFFERENTIATION FACTOR WITH APPARENT PROLIFERATION ACTIVITY ON ACTIVATED B CELLS

Swain, Dutton et al (49, 50) recently found B-cell growth factor (BCGF-II) in CFS from antigen-activated T-cell clones or T-cell hybridomas. According to their findings, BCGF-II activity can be assayed in a costimulator assay with normal B cells and dextran sulfate or with the BCL-1 in vivo line. Moreover, CFS of B151K12 exerted potent BCGF-II activities on BCL-1 cells.

To examine whether B-cell growth-inducing activity is mediated by the same TRF molecule responsible for inducing differentiation of activated B cells, we undertook purification of the B151-TRF molecule (51). The purification scheme consisted of ammonium sulfate precipitation, DEAE-cellulose chromatography, Blue-Sepharose chromatography, hydroxyl-apatite chromatography, gel permeation with fast protein liquid chromatography (FPLC), and disc polyacrylamide gel electrophoresis. Overall, B151-TRF was purified approximately 34,000-fold with a maximum 3.8% recovery of activity. During the course of the purification procedure, BCGF-II activity was simultaneously evaluated. BCGF-II activities always resided in the same fraction in which B151-TRF activity was detected. Physicochemical characterization of B151-TRF revealed that the active molecule is hydrophobic, with M_r 50,000–60,000 on gel permeation chromatography and 18,000–19,000 on SDS-PAGE. Highly purified B151-TRF activity was lost after treatment with trypsin but not with RNase. Moreover, it bound to lima bean agglutinin (LBA)-Sepharose, indicating that B151-TRF is a glycosylated protein containing N-acetylgalactosamine residues. Further detailed analyses convincingly demonstrated that B-cell growth and differentiation activity of B151 supernatant is mediated by a single TRF molecule (52). This conclusion was derived from the following sets of observations: (*a*) B-cell growth and differentiation factor (BGDF) activity was copurified by reversed-phase HPLC and LBA-agarose column. (*b*) This active fraction revealed a single band of 19kd by SDS-PAGE and autoradiography analysis by utilizing ^{225}I-labelled material. (*c*) The induction of B-cell growth and differentiation by this molecule was blocked comparably by adding N-acetylgalactosamine in the culture. Thus, these studies indicated that a single TRF molecule induces a certain

population of activated B cells to undergo both clonal expansion and differentiation into Ig-secreting cells.

B-CELL DIFFERENTIATION FACTOR WITHOUT APPARENT PROLIFERATION ACTIVITY ON ACTIVATED B CELLS

One aspect of factor-mediated regulation of B-cell differentiation still requiring significant exploration is whether or not all the B-cell differentiation factors are capable of stimulating proliferation. Indeed, this characterization is hard to resolve, since it is unclear whether or not B cells require one or several cycles of division before producing antibody. However, this may not be a crucial issue for understanding the mechanisms of B-cell differentiation governed by the T cells or their products. Rather, this distinction may provide us with information on how many factors are involved in the consecutive sequence of B-cell differentiation and, secondly, how different these factors are from each other with respect to the molecular mechanisms of B-cell activation.

Kishimoto and colleagues (53) have reported BCDF-secreting human T-cell hybridomas that secrete a factor that does not support B-cell proliferation. The BCDF produced by their newly HTLV-transformed human T-cell line (TCL-NaI) had a M_r of 35,000 and a pI value of 5.5. This distinguishes BGDF, which was eluted in the fractions corresponding to M_r of more than 60,000 and pI values of 5 to 6 (54).

In the murine system, EL-TRF may represent a B-cell differentiation factor that lacks proliferation activity on activated B cells. In concert with B151-TRF, EL-TRF acts to drive anti-Ig-activated B cells into Ig-secreting cells (21, 41). The EL-TRF is distinguished, as mentioned previously, by kinetic analyses showing that EL-TRF acts at a much later stage of an Ig-synthesizing response (i.e. day 3 versus day 2 of B151-TRF of a 4-day anti-IgM-induced response). EL-TRF does not act in either the anti-IgM or the dextran sulfate synergy proliferation. Biochemical studies indicate that EL-TRF is trypsin-sensitive, has an approximate M_r of 30,000–40,000 by gel filtration analysis, an isoelectric point of 4.5, and is moderately hydrophobic as determined by phenyl Sepharose chromatography (43).

IL-2 AND GAMMA-INTERFERON AS DIFFERENTIATION FACTORS ON ACTIVATED B CELLS

It has been reported that interleukin 2 (IL-2) also expresses its function as a B-cell differentiation factor under certain experimental conditions (55–59).

The observation that EL-TRF activity was found in the pH 4.5 fraction of an isoelectric focusing separation, close to the pI of IL-2 (43), raised the question as to whether IL-2 is also a B-cell differentiation factor. In fact, EL-4 culture supernatant contains two molecular species (EL-TRFs) that have differentiative activity. One comigrates with IL-2, and its activity is blocked by antibody to the IL-2 receptor. IL-2 was found to have EL-TRF activity, by measurement of the induction of cytoplastic IgM, and also caused an 8–16-fold increase in levels of mRNA for the secretory form of μ heavy chains when used on B cells cocultured with anti-IgM, BSFp1, and B151-TRF. An important observation relating to the action of IL-2 as an EL-TRF is that high concentrations (100 U/ml or above) of this lymphokine are required for induction of IgM synthesis. The other product with EL-TRF activity has an M_r of 32,000. This material lacks IL-2 and γ-interferon (IFN) activity. Moreover, antibody to the IL-2 receptor does not impair this EL-TRF function (43).

Results from some other recent studies have also suggested that γ-IFN may be a component of TRF. Indirect evidence was obtained from the acid-labile character of TRF present in antigen- or Con A-stimulated–T-cell supernatant (60, 61) and also from a correlation of γ-IFN levels and TRF activity of supernatant of T-cell hybridomas (62) in some systems. This view is further supported by recent data (63, 64) showing that recombinant DNA-derived murine γ-IFN can act as a second component of a complementing TRF system to support PFC responses to antigen (63). Thus, the data suggest that purified γ-IFN may modulate B cells in their maturation to antibody production.

Kishimoto and colleagues (65) also recently reported the effects of IL-2 and γ-IFN on the activation of human B cells with recombinant IL-2 and γ-IFN. BCDF-responsive B-lymphoblastoid cell lines and highly purified human B cells were employed as target B cells. IL-2 or γ-IFN did not induce any IgG or IgM secretion in the B-cell lines CESS and SKW6-CL4, in which IgG and IgM were inducible with conventional T-cell factor(s). IL-2 alone did not induce the optimum production of Ig but did induce proliferation in the Staphylococcus Cowan I (SAC)–stimulated B-cell population. The addition of γ-IFN together with IL-2 induced IgM and IgG secretion that was comparable with that induced by a conventional T-cell factor(s). IL-2 induced proliferation not only in SAC-stimulated B cells but also in an anti-μ-stimulated B-cell population. Double staining of anti-μ-stimulated B cells with anti-Ig and anti-TAC antibodies demonstrated that anti-μ stimulation induced an increased expression of TAC antigen on surface Ig-positive B cells.

All of these results strongly support the notion that IL-2 can act as a growth factor for B cells, and γ-IFN can mediate differentiation of B cells.

However, it is not clear to what extent this is accomplished through the pathway normally regulated by TRF.

B-CELL DIFFERENTIATION FACTOR ACTING ON RESTING B CELLS

One of the major issues regarding activation of B lymphocytes is whether resting B cells which usually require cognate interaction with T cells for their activation can be directly stimulated by certain lymphokines to differentiate into antibody-secreting cells. As summarized above, a recent model describing B-cell activation by lymphokines (21, 22) proposes that anti-Ig and B-cell growth factors synergize to induce polyclonal proliferation of resting B cells. B-cell differentiation factors then induce Ig secretion by the activated cells. Although most published data are compatible with this model, they do not exclude the possibility that different lymphokines act on different subsets of B cells, rather than sequentially on the same B cells. There is also controversy as to whether lymphokines exist that can move a resting B cell in stage G0 to Ig-secretion.

B-Cell Maturation Factor

Some T cell–derived factors have the ability to activate resting G0-phase B cells to undergo terminal differentiation. Melchers and colleagues (66) reported that although small B cells (isolated on the basis of cell density) could not proliferate in response to B-cell stimulation factor, they could differentiate into Ig-secreting cells. B-cell activation is characterized by a transition from the G0 to G1 phase of the cell cycle and by cellular enlargement. In their system, a factor with M_r of 30,000 was isolated from the supernatant of a helper-T-cell line specific for horse erythrocytes. This factor has been designated as B-cell replication and maturation factor. Leclercq and associates (67) also reported that helper-T-cell supernatant is sufficient to induce a polyclonal proliferation and differentiation of resting B cells. The differentiation of resting B cells in the presence of T-cell supernatant may operate through a pathway other than the sequential one summarized earlier and may be mediated by some unique lymphokine(s).

Sidman and colleagues also recently identified a B-cell differentiation factor acting on the resting B cells and designated as B-cell maturation factor (BMF). BMF appears to cause polyclonal Ig secretion of normal B cells in high frequency with virtually no, or only marginal, proliferation. The responding B cells include neonatal B cells, B cells from various organs, nu/nu B cells, and B cells from CBA/N mice. Response of B cells separated by Percoll density gradients or 1 g sedimentation velocity gradients indicates that BMF acts upon small resting B cells.

According to Sidman and colleagues, BMF is a mildly acidic (pI 5–6) and extremely hydrophobic glycoprotein, with an apparent M_r of 50,000–55,000 daltons on gel permeation and 16,000 of SDS-PAGE (68, 69).

B151-TRF 2

Recently we have found another unique B-cell differentiation factor acting on resting B cells in the supernatant of B151-T hybridoma (S. Ono, S. Hayashi, Y. Takahama, K. Dabashi, Y. Kato, K. Nakanishi, W. E. Paul, T. Hamaoka, manuscript in preparation). Further detailed analyses indicate that the B151 supernatant factor responsible for the differentiation-inducing activity on resting B cells is distinct from the previously described B151-TRF, which causes growth and differentiation of activated B cells. [Thus, the B151-TRF described in the section above can be renamed B151-BGDF according to the new system of nomenclature (39, 21, 22). For reasons of simplicity, however, we will refer to this as B151-TRF 1.] The newly found factor activates resting splenic B cells prepared by Percoll density centrifugation, as well as neonatal B cells, with a striking increase in IgM-secreting cells in 4- or 5-day culture without antigenic stimulation. (Thus, this factor can be designated as B151-BCDF. We will refer to this new factor as B151-TRF 2.) Approximately 10–20% of the total B cells added to the culture responded to this factor. Loss of B151-TRF 2 activity by treatment with either Sepharose-bound proteolytic enzymes or heating at 56°C for 30 min indicated that the activity is mediated by protein. The response of C3H/HeJ and B10ScCr B cells (which are low or nonresponders to lipopolysaccharide) strongly argues against the possibility that the activity of B151-TRF 2 is mediated by lipopolysaccharide contamination of culture medium. Although B151-TRF 2 induces differentiation of resting B cells, this factor is distinct from the Sidman's BMF. The differences were determined by the following observations: (*a*) B151-TRF 2 is a rather acidic (pI 4.3–4.5) and extremely hydrophobic (more than B151-TRF 1) protein without detectable sugar, as is revealed by the lack of significant binding to various lectin-columns. The apparent M_r was 30,000–35,000 on gel permeation (Y. Kato, S. Ono, Y. Takahama, T. Hamaoka, manuscript in preparation). This is in sharp contrast with the 50,000–55,000 M_r glycoprotein nature of BMF. (*b*) CBA/N B cells are nonresponders to B151-TRF 2. This is again in sharp contrast to the positive response of CBA/N B cells to BMF. Interestingly, DBA/2Ha B cells, which are nonresponders to B151-TRF 1 (70) and BMF (Sidman; personal communication), respond quite well to B151-TRF 2.

The B151-TRF-1 and B151-TRF 2 activities could be selectively absorbed with BCL1 and DBA/2Ha B cells, respectively. These cell lines were selected by the preferential expression of the TRF 1 and TRF 2 receptors on their

surface. Moreover, TRF 2 activity revealed specific binding-affinity to N-acetylglucosamine-coupled agarose beads but not to any lectin-coupled beads so far tested, whereas TRF 1 activity showed no binding to sugar-coupled bead but did bind to the LBA column. These results, in conjunction with the preferential inactivation of TRF2 activity by heat treatment (56°C, 30 min) without affecting TRF-1 activity, clearly indicate that the activities are mediated by two distinct molecules. An important conclusion drawn from these results is that resting B cells can be activated by soluble T-cell factors in the absence of any overt signal through the Ig receptor by anti-Ig-like stimuli.

Whether and how these factors described above are active in MHC-restricted cognate T-T cell interactions or mediate some other completely different pathway of B-cell activation has not yet been determined, however. It can be anticipated that rapid progress in the understanding of the receptor molecule involved in the interaction of those lymphokines on the B-cell activation, part of which we discuss later, will solve this issue in the next few years. In Figure 1 and Table 1, the various pathways of B-cell differentiation and factors involved are summarized. In conclusion, several

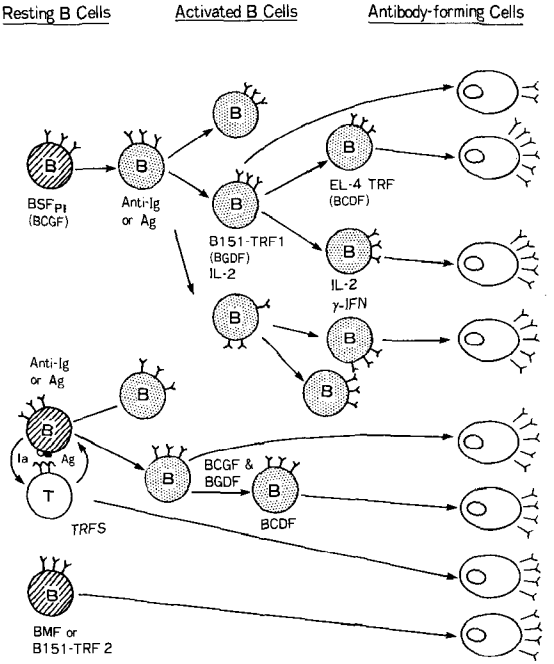

Figure 1 Schematic illustration of B-cell differentiation process and signals required for the B-cell activation. BCGF, B-cell growth factor, BGDF, B-cell growth and differentiation factor; BCDF, B-cell differentiation factor.

Table 1 Murine B-cell differentiation factors

Responding cells	Murine B-cell differentiation factor (activity)	Molecular weight	Other properties
Ag or anti-Ig activated B cells	B151-TRF 1 (BGDF)	50,000–55,000 (gel-filtration) 18,000–19,000 (SDS-PAGE)	Glycoprotein PI 5.0 Responder (CBA/N and others)
	EL-4 TRF (BCDF)	30,000–40,000 (gel-filtration)	PI 4.5
	IL-2 (BGDF) γ-interferon (BCDF)		
Resting B cells	B-cell replication and maturation factor (BCDF)	30,000 (gel-filtration)	
	BMF (BCDF)	50,000–55,000 (gel permeation) 16,000 (SDS-PAGE)	Glycoprotein PI 5-6 Responder (CBA/N and others)
	B151-TRF 2 (BCDF)	30,000–35,000 (gel filtration)	Protein PI 4.3–4.5 Responder (DBA/2Ha and others) Nonresponder (CBA/N)

B-cell differentiation factors have been defined. Some of these molecules differ from each other on the basis of functions and physicochemical properties.

RECEPTORS FOR FACTORS INVOLVED IN THE B-CELL DIFFERENTIATION

As mentioned above, several B-cell differentiation factors have been demonstrated, and B-cell differentiation signals vary considerably depending on the nature of the B-cell subpopulation under study. The differences in factor-responsiveness of B cells may be reflected by the difference in expression of the functional receptors on the same lineage of B cells to respective B-cell differentiation factors, or they may be due to the different pathways of B-cell activation that occur in B cells of distinct lineages. For further analysis of this point, more information on the receptors corresponding to the B-cell differentiation factors is required.

IL-2 Receptor

Among the numbers of putative receptors of B cells to various lymphokines, the IL-2 receptor represents one of the best characterized molecules. This characterization has been facilitated by the availability of anti-IL-2 receptor monoclonal antibody and recombinant IL-2. In general, activated B cells express small numbers of membrane molecules that bear epitopes in common with the T-cell IL-2 receptor, and activated B cells bind small numbers of IL-2 molecules (71–75). Nakanishi et al (43) demonstrated that B cells stimulated with anti-IgM and BSFp1, with or without B151-TRF 1, express determinants that react with two monoclonal antibodies that recognize distinct epitopes on the T-cell IL-2 receptor. These determinants were present at much lower density (100-fold) on stimulated B cells than on activated T cells. Indeed, similar ratios were obtained by comparing numbers of molecules of ^{3}H IL-2 bound by activated T cells and by stimulated B cells with the relative binding of monoclonal anti-IL-2 receptor antibodies by these two cell populations. Both approaches suggest that activated T cells have 56–125-fold more IL-2 receptors than B cells stimulated with anti-IgM, BSFp1, and B151-TRF 1. The action of IL-2 in causing activated B cells to differentiate to high rate IgM-synthesizing cells was dependent upon their treatment with B151-TRF 1. Without B151-TRF 1, neither enhancement in levels of mRNA for the secretory form of μ-heavy chains nor the presence of cells with cytoplasmic IgM was observed. However, B cells cultured with anti-IgM and BSFp1, without B151-TRF 1, did express epitopes recognized by anti-IL-2 receptor antibodies and these stimulated B cells could bind small amounts of ^{3}H IL-2. Thus, the expression of IL-2 receptors on B cells does not appear to depend upon the presence of B151-TRF 1.

The binding of anti-IL-2 receptor antibodies to B cells activated with anti-IgM and BSFp1, with or without B151-TRF 1, is perhaps not surprising. Mouse B-cell blasts induced with lipopolysaccharide have already been shown to bind anti-IL-2 receptor antibody (76). Human hairy cell leukemias, which are a form of B cells as judged by Ig gene rearrangement, and some human lymphoblastoid cell lines, as well as activated B cells, have been shown to possess the TAC antigen (77), which is an epitope on the human T-cell IL-2 receptor. Thus, the action of IL-2 as EL-TRF, its inhibition by anti-IL-2 receptor antibody, the presence of the epitope on activated B cells, and the capacity of activated B cells to bind small amounts of ^{3}H IL-2 are all in keeping with a direct action of IL-2 on B cells that have been cultured with anti-IgM, BSFp1, and B151-TRF 1. Using a single hapten-specific B-cell culture in the absence of any feeder, filler, or accessory cells, Pike et al (78) reported that a B cell could be

stimulated to division and differentiation only in the concomitant presence of lymphokines acting as B-cell growth and differentiation factor (BGDF). In this system, human IL-2 prepared by recombinant DNA technology was also effective as BGDF, albeit with rather weak activity. However, when an IL 2–free source of BGDF was used with the antigenic stimulus, addition of IL-2 did not augment the response, nor did removal of IL-2 from the crude lymphokine mixture diminish the BGDF activity.

Structurally, the IL-2 receptor found on activated B cells is identical with that on activated T cells, since anti-TAC antibody immunoprecipitated a molecule of identical size (M_r 65,000) from T and B lymphocytes. B cells were also shown to synthesize the IL-2 receptor actively. Moreover, the chymotryptic peptide chromatograms of TAC antigen from T and B cells show these molecules to be indistinguishable (75, 80, 81).

B151-TRF 1 Receptor and B151-TRF 2 Receptor

B151-TRF 1 and -TRF 2 both stimulate syngeneic and allogeneic B cells, indicating that B151-TRFs trigger B cells irrespective of the major histocompatibility type. Importantly, antigen-primed but not antigen-nonprimed B cells from X-linked defective CBA/N mice responded well to B151-TRF 1, whereas B cells from the mutant mice of DBA/2Ha strain failed to show any significant response to the B151-TRF 1 even after antigen-priming (82). The responsiveness of the B151-TRF 1 is controlled by the gene linked to the X chromosome, which is defective in DBA/2Ha mice. In contrast, with respect to the responsiveness to B151-TRF 2, DBA/2Ha B cells are high responder and CBA/N B cells are nonresponder.

In order to examine whether the absence of a particular receptor site for TRFs on low responder B cells determines their response type, we conducted experiments to measure the ability of B cells from high responder and low responder mice to absorb TRFs' activities. Residual TRFs' activities after absorption with those B cells were determined. The results demonstrated that B cells from BALB/c mice were able to absorb both TRFs' activities. In contrast, B cells from DBA/2Ha mice could absorb TRF 2 activity but not absorb the TRF 1 activity, indicating the lack of a particular functional receptor site for TRF 1. In sharp contrast to this, BCL-1 cells could absorb TRF 1 activity but not TRF 2 activity. Interestingly, CBA/N B cells could absorb both TRF 1 and TRF 2 activities, whether or not the B cells were primed with antigen. This indicates that the defective response of CBA/N B cells to B151-TRFs is not determined by the absence of corresponding receptors but may be due to a defect in some undefined signal transmission mechanism(s), which are discussed later.

By taking advantage of the selective absence of TRF 1 receptor on

DBA/2Ha B cells, an antiserum was raised in (DBA/2Ha × BALB/c) $(DC)F_1$ male mice by immunization with BALB/c B cells or BCL-1 cells, and then it was evaluated. It was found that DCF_1 male anti-BALB/c B-cell antiserum specifically inhibited the B-cell responses mediated by the B151-TRF 1 in the continuous presence of antiserum in the culture. Furthermore, the suppressive activity of this antiserum was successfully removed by absorption with high responder BALB/c B cells and BCL1 cells but not with low responder DBA/2Ha B cells (82). Moreover, this antiserum augmented primary IgM anti-sheep red blood cell (SRBC) antibody responses when intravenously injected in combination with suboptimal doses of antigen in BALB/c as well as CBA/N mice, but not in DBA/2Ha mice (83–85). Thus, the DCF_1 male anti-BALB/c B-cell antiserum contains antibodies capable of substituting for TRF activity. The ability of this antibody to substitute for TRF 1 activity was also obvious in the in vitro secondary anti-DNP IgG PFC responses (86). In this system, the anti-Thy 1 plus C-treated DNP-primed B cells from B151-TRF 1 high-responder mice were effectively stimulated by this antibody, whereas B cells from low-responder DBA/2Ha mice were triggered only with difficulty by the antibody under the same conditions. An intriguing finding was that the DNP-primed B cells from CBA/N mice were also effectively triggered by this antibody, suggesting that the putative TRF 1 receptor detectable by this antibody is distinct from Lyb-3 and Lyb-5 molecules, which are defined to be lacking in CBA/N mice.

In order to study the mechanism of B-cell triggering via the B151-TRF 1 receptor, we examined the TRF 1–substituting activity of the $F(ab')_2$ and Fab′ fragments of the DCF_1 male anti-BALB/c B cell antibody in the induction of secondary IgG PFC responses (86). The results showed both the bivalent $F(ab')_2$ and monovalent Fab′ fragments of the anti-TRF 1 receptor antibody activity were comparable to the IgG fraction in inhibiting TRF-mediated anti-DNP IgG PFC responses when added continuously to the culture. This indicates that these antibody preparations possess binding activity operationally comparable to the TRF 1 receptor. On the other hand, the monovalent Fab′ alone possessed no ability to stimulate B cells. This was indeed in sharp contrast to the potent TRF 1–substituting activity of the $F(ab')_2$ and IgG. Thus, these results indicate that the bivalent anti-TRF 1 receptor antibody can lead to B-cell triggering. The molecular and cellular mechanisms of this stimulation are under study.

Data from these experiments provided several important insights into the mechanism of TRF 1 action. The first is that many, and perhaps all, of the lymphokine actions in the induction of B-cell differentiation can be initiated by the interaction of ligands other than the lymphokine with the

receptor molecule. The data suggest that the TRF 1–receptor perturbation by ligand or anti-receptor antibody, when properly triggered, is capable of initiating B-cell differentiation. The second major point is that receptor aggregation or clustering by ligand is required for the effective induction of B-cell differentiation. However, this does not necessarily imply that the TRF 1 molecule itself has the bivalent binding sites to the receptor. It can be postulated as a possibility that, even if TRF 1 is a monovalent molecule, the crosslinking of the receptor could be induced by some serum protein–bound form by virtue of its hydrophobic property.

The further detailed chemical properties of the B151-TRF 1 receptor molecule have been summarized in Hamaoka (87), and some intriguing functional properties of TRF 1 receptor and TRF 2 receptor are further discussed in the following section.

ROLE OF SUGAR MOIETY IN THE INTERACTION OF B-CELL DIFFERENTIATION FACTORS WITH CORRESPONDING RECEPTORS

Several studies on the role of carbohydrate moieties in lymphoid cell interactions have suggested the involvement of carbohydrates in the triggering of lymphoid cell functions, e.g. mannose blocking of the T cell–macrophage interaction (88) and appearance of alloantigen-presenting activity of B lymphocytes after sialidase-treatment (89). Some functional studies on lymphokines suggested that carbohydrate structures on the receptors for lymphokines are responsible for the expression of their functions. These are exemplified by the possible role of α-L-fucose on migration-inhibitory factor receptor (90), galactose residue as the lymphotoxin binding site (91), and ganglioside GM_2 as an interferon binding site (92). On the other hand, direct evidence for the functional significance of carbohydrate moiety on lymphokine molecules has not been clearly demonstrated. Studies on structures of lymphokines have identified some carbohydrate residues on lymphokine molecules. Robb et al (93) reported the existence of O-linked N-acetylgalactosamine-containing carbohydrate structure on the interleukin 2 (IL-2) molecule. However, in this case no change was observed in the IL-2 activity even in the absence of sugar moiety. Ishizaka and colleagues (94, 95) demonstrated that N-linked carbohydrate structure on IgE-binding factor determined its activity. IgE-binding factor glycosylated by glycosylation-enhancing factor potentiated IgE production, whereas inhibition of its glycosylation by glycosylation-inhibitory factor converted it into IgE-suppressive factor. However, in these studies, the role of carbohydrate residues in the expression of their activities has not yet been clarified.

Role of Gal-NAc Moiety on B151-TRF 1 Molecule in Its Binding to TRF 1 Receptor and B-Cell Differentiation

Concerning the role of sugar moiety for the B-cell differentiation factor activity, particularly interesting is the description by Tomaska & Parish (96) of the inhibition with Gal-NAc of Concanavalin A–induced, T cell factor–mediated, B-cell differentiation of DNP-primed B cells. In accordance with their results, Takahama et al in our laboratory (97) demonstrated that the induction of B-cell differentiation into Ig-secreting cells by B151-TRF 1 was specifically inhibited by addition of N-acetyl-D-galactosamine (Gal-NAc) to the culture. Such inhibition appeared to be the interference of Gal-NAc with the binding of TRF1 to its receptor, since absorption of TRF 1 activity with B cells was notably inhibited by the presence of Gal-NAc. We also established a binding assay of B151-TRF 1 molecule to the receptor on B cells using radio-labelled TRF 1 fraction, and we demonstrated that the binding of B151-TRF 1 molecule to the receptor-positive B cells such as BCL1 was almost abrogated by Gal-NAc. Moreover, the existence of Gal-NAc residue(s) on B151-TRF 1 molecule was determined by the fact that the TRF 1 molecule specifically bound to the lectin-gels, such as LBA-, DBA-, SBA-, and LPA-coupled agarose beads. The functional significance of the Gal-NAc moiety in the expression of B151-TRF 1 activity was also supported by the fact that TRF 1 activity disappeared after treatment of B151-TRF 1 with α-N-acetyl-galactosaminidase. Although sialic acid residue(s) were also detected on B151-TRF 1 molecule, their functional role is obscure. Thus, the Gal-NAc residue(s) on B151-TRF 1 molecule play an important role in binding of TRF 1 molecule to the receptor and in the induction of B-cell differentiation. Since the induction of B-cell differentiation by conventional TRF 1 activity in antigen-primed T-cell culture supernatants was also inhibited by adding Gal-NAc to the culture, inhibition with Gal-NAc seems to be a general phenomenon in TRF 1–mediated B-cell differentiation.

Receptor molecules with carbohydrate-binding properties have been demonstrated previously, notably the asialoglycoprotein receptor on liver cells (98) and the mannose-6-phosphate receptor on fibroblasts (99). Since Gal-NAc residue(s) on B151-TRF may be recognized by TRF-receptor on B cells, it is of interest to examine whether or not Gal-NAc binding to the TRF-receptor is enough to activate B cells. Our preliminary results revealed, however, that neither Gal-NAc-coupled bovine serum albumin nor Gal-NAc-coupled agarose beads was able to stimulate B cells, even though both reagents demonstrated significant binding to the TRF 1–receptor-positive BCL-1 cells. Therefore, it is likely that in addition to Gal-

NAc moiety, another oligosaccharide portion or a portion of polypeptide may be also required for the expression of TRF 1 activity.

Glc-NAc-Binding Property of B151-TRF 2 Molecule and Its Role in TRF 2–Receptor Binding and B-Cell Differentiation

In contrast to the role of Gal-NAc moiety on B151-TRF 1 molecule, the specific affinity of B151-TRF 2 molecule to the N-acetylglucosamine (Glc-NAc) plays an important role in the stimulation of resting B cells. As briefly summarized in the above section, B151-TRF 2 is a heat (56°C, 30 min)-labile nonglycosylated protein, stimulatory to the B151-TRF 1–unresponsive DBA/2Ha B cells. Interestingly, however, the addition of Glc-NAc (20–40 mM) to the culture of a TRF 2 assay strikingly inhibited the induction of Ig-secreting cell response from the resting B cells. Indeed, this was mediated by the specific reaction of Glc-NAc to the TRF 2 response, since the TRF 1 response induced to the activated B cells was not inhibited under this condition.

In order to establish that the inhibition of B151-TRF 2 response by Glc-NAc is mediated by the blocking of receptor-ligand interaction, inhibition of absorption of B151-TRF 2 activity with B cells by the presence of Glc-NAc was demonstrated. In addition, Glc-NAc-binding property of B151-TRF 2 was demonstrated by its binding to the Glc-NAc-agarose beads and specific elution from the column with Glc-NAc solution. The existence of Glc-NAc moiety on the TRF 2 receptor and its involvement in ligand-receptor interaction were further substantiated by the fact that N-acetyl-glucosaminidase(Glc-NAc-ase)-treatment of B cells specifically induced a loss of their ability to absorb B151-TRF 2 activity, whereas the absorbing activity of the same B cells for B151-TRF 1 activity was not changed. Moreover, the effect of Glc-NAc-ase treatment on B cells was specifically inhibited by the presence of p-nitrophenyl Glc-NAc. Finally, we examined whether the binding of Glc-NAc-specific lectin could substitute for the B151-TRF 2 activity and activate resting B cells. The results revealed that Glc-NAc-specific, wheat germ agglutinin–bound agarose beads with appropriate density significantly stimulated the resting B cells (Y. Kato, S. Ono, Y. Takahama, T. Hamaoka, manuscript in preparation). Taken collectively, these results indicate that B151-TRF 2 molecules interact with the corresponding receptor via the recognition of Glc-NAc moieties on the receptor.

Thus, in conclusion, a series of these studies clearly illustrated the existence of a carbohydrate-dependent signal transmission mechanism in the B-cell differentiation process mediated by the factor. The molecular

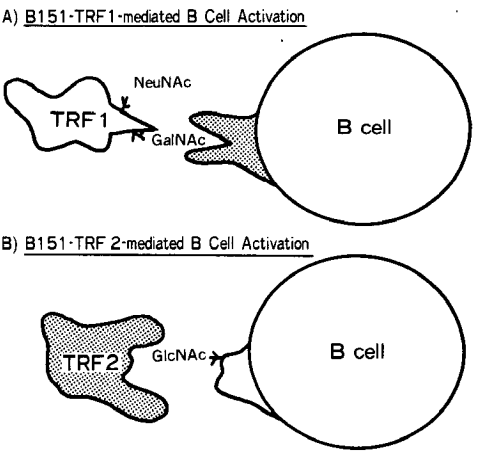

Figure 2 Ligand-receptor interaction in B151-TRF 1– and B151-TRF 2–mediated B-cell differentiation. NeuNAC, Sialic acid.

properties of B151-TRF 1 and -TRF 2 and their mode of interaction with corresponding receptors are summarized in Table 2 and schematically depicted in Figure 2.

BIOLOGICAL SIGNIFICANCE OF B151-TRF 1 AND -TRF 2 MOLECULES IN B-CELL DIFFERENTIATION

Most of lymphokine-producing cell lines have been propagated over long periods of time. Because continuously cultured cell lines have an extremely high incidence of mycoplasma contamination, and because several mycoplasma species can induce the proliferation and differentiation of both human and murine B cells (100–102), we should exclude, therefore, the possibility that B-cell differentiation factor, found in supernatant of B151-T hybridoma, or from some other T-cell lines as well, may be due to mycoplasma or some other unknown microorganism contamination.

B151-TRF 1 as a Physiological Mediator of Terminal B-Cell Differentiation

Nordin and associates (103) demonstrated that a soluble product with B-cell stimulating activity was separated from the serum-free supernatant of a T-cell hybridoma infected with mycoplasma. Hybridoma cells freed of mycoplasma by detergent treatment failed to produce active supernatant, and reinfection of the treated cells reconstituted the activity. Furthermore,

Table 2 Properties of two distinct TRF molecules from B151-T-cell hybridoma

Properties	B151-TRF 1	B151-TRF 2
Responding cells	Ag-primed B cells Activated B cells (not DBA/2Ha B cells) BCL1 cells	Neonatal B cells Adult resting B cells DBA/2Ha B cells (not BCL1 cells)
T-cell or accessory-cell dependency	No	No
M_r (gel-filtration)	50,000–55,000	30,000
(SDS-PAGE)	19,000	ND
Proteinase treatment	Sensitive	Sensitive
Heat stability (56°C, 30 min)	Stable	Unstable
Preferential binding	BCL1 cells	DBA/2Ha B cells
Lectin-gel binding	LBA, DBA, LPA, SBA	None
Sugar moiety	Gal-NAc, Sialic Acid	None
Inhibitable by	Gal-NAc	Glc-NAc
Sugar-gel binding	None	Glc-NAc-gel

ND, not done.

deliberate infection of a mycoplasma-free unrelated T-cell hybridoma, as well as the monocytic cell line, resulted in the production of supernatants with B-cell stimulation activity.

The facts that at least B151K12-T hybridoma is free of any detectable mycoplasma and that the molecular weight of the reported active fraction (average of 90,000) is different from B151-TRF 1 as well as from -TRF 2 seem to negate the possibility that these factors are mycoplasma-derived soluble products. However, these are still not formal proof that B151-TRF 1 and -TRF 2 are the physiological mediators of B-cell differentiation.

Previously, we demonstrated that antigen (*Mycobacterium tuberculosis*; Tbc)-primed helper T cells of the surface phenotype Lyt $1^+, 2^-, 3^-$ released soluble factor(s) upon stimulation with purified protein derivative (PPD), which replaced T-cell activity in the induction of the secondary anti-DNP IgG-PFC response to soluble DNP-carrier conjugates (104). This TRF 1-responsivity of B cells is under X-chromosome control, and DBA/2Ha B cells are nonresponder to the TRF 1. By use of this nonresponder characteristic of DBA/2Ha B cells, the B-cell differentiation factor that was released from T-cell hybridoma was screened, and the B151K12 clone was selected (42).

The B151-TRF 1 obtained from a continuously cultured B151K12 clone acts on the TRF 1–receptor site on B cells according to the same mechanism as seen with PPD-induced TRF from Tbc-primed T cells. This was demonstrated by the fact that the alloantiserum raised in the low-responder (DBA/2Ha × BALB/c)F_1 male mice by immunization with high-responder parental BALB/c B cells amply blocked B-cell triggering mediated by B151-TRF 1 (42). Furthermore, as mentioned earlier, GalNAc inhibited both PPD-induced TRF 1 and B151-TRF 1 activities. These results, taken together, indicate that the B151-TRF 1 acts on the same physiological TRF 1 receptor on B cells as does the antigen-induced TRF 1.

Preferential Induction of TRF 2 Activity from T Cells of Autoimmune MRL/lpr Mice

The biological significance of B151-TRF 1 and -TRF 2 for the B-cell differentiation was further demonstrated by the fact that this activity was also detectable under certain pathological conditions associated with the polyclonal B-cell stimulation, i.e. in the chronic graft-versus-host reaction (GVHR) as well as in the MRL/lpr mice with T cell–proliferative autoimmune disease. Prud'homme et al (105, 106) reported that lymph node and spleen cells of the autoimmune MRL/Mp-lpr/lpr mouse spontaneously produce (in the absence of any mitogenic or antigenic stimulation) one or more B-cell differentiation factors that induce or enhance polyclonal B-cell differentiation and Ig-secretion. This factor is not produced by the congenic MRL/n mouse strain that lacks the lpr gene. Moreover, lymphoid cells of the B6-lpr/lpr strain also produce the similar B-cell differentiation factor. The factor acts on resting B cells. Cell depletion studies revealed that this factor is produced by T cells of the Lyt-1^+2^- phenotype. Because of its association with the lpr/lpr genotype, they term this B-cell differentiation factor L-BCDF. Functional analysis of L-BCDF revealed that it induces B-cell differentiation in the absence of IL-2 and BCGF. No T cell proliferation–inducing activity or B cell growth factor–like activity can be detected in MRL/lpr-derived supernatants.

The above studies did not establish, however, whether L-BCDF is a single factor or a combination of factors. L-BCDF by itself did not have conventional TRF (TRF 1) activity. Namely, L-BCDF did not restore the primary in vitro anti-SRBC response of T cell–depleted splenic B cells (105). We functionally compared our B151-TRF 1 and -TRF 2 activities with the above B-cell differentiation factor(s) and tried to determine any biological resemblance (K. Dobashi, S. Ono, S. Murakami, T. Hamaoka, manuscript in preparation). The results demonstrated that the 24 hr–culture supernatants of the spleen and lymph node cells from MRL/lpr strain (but not

from MRL/n strain) contained B151-TRF 2–like activity in our assay. The B-cell differentiation factor activity of the MRL/lpr culture supernatant as the mediation of TRF 2 was implicated by the following observations: (*a*) Both B151-TRF 2 and L-BCDF did activate resting B cells to differentiate into Ig-secreting cells without antigenic stimulation; (*b*) the B151-TRF 2–like activity in L-BCDF was almost completely absorbed by DBA/2Ha B cells but not by BCL-1 cells, which were established to express B151-TRF 2 and -TRF 1 receptors, respectively; (*c*) the TRF 2–like factor activity in MRL/lpr supernatant was inactivated by the heat treatment at 56°C for 30 min; (*d*) the differentiation of resting B cells by MRL/lpr supernatant was significantly blocked by Glc-NAC but not by Gal-NAC; (*e*) finally, the B-cell response induced by MRL/lpr factor could be completely inhibited by the addition of prostaglandin E2 to the culture, in a manner analogous to the B151-TRF 2–induced B-cell differentiation. These results strongly support the notion that B151-TRF 2 represents at least a part of L-BCDF. In fact, the injection of B151-TRF 2 into normal BALB/c mice induced a striking PFC response against bromelain-treated (Bm) mouse red blood cells (MRBC), representing one of the autoantibody responses, and this activity was indeed heat labile. Especially the latter observation reinforces the notion that B151-TRF 2 is a physiological substance responsible for the polyclonal stimulation of B cells, particularly in the induction of auto-antibody responses.

TRF 2 as a Polyclonal B-Cell Stimulator for Autoantibody-Induction in Chronic GVHR

In addition to the spontaneous lupus models described above, auto-antibody production and an SLE-like disease can also result from the induction of chronic graft-versus-host(GVH) disease across I-A or I-E (but not usually H-2D or H-2K) differences. In this case, donor-T-cell proliferation is associated with stimulation of the recipient's B cells, leading to polyclonal B-cell stimulation, production of anti-DNA and other auto-antibodies, and immune complex disease (107–109).

In contrast to the preferential expression of TRF 2 activity in the MRL/lpr supernatant, the culture supernatant of spleen cells from mice with chronic GVHR disease, i.e. (C57BL/6J × DBA/2J)F_1 transferred with DBA/2J spleen cells, produced both TRF 1- and TRF 2-activities. It is interesting, however, that the spleen cells of (C57BL/6J × DBA/2J)F_1 mice transferred with DBA/2J thymocytes preferentially produced TRF 1–like activity. The TRF 1–like activity detected under these conditions was almost completely absorbed by BCL-1 cells, but not by DBA/2Ha B cells; it was heat resistant and blocked by Gal-NAC, but not by Glc-NAC.

Moreover, the B-cell differentiation induced by TRF 1–like activity in this culture supernatant was completely resistant to prostaglandin E2, and this was indeed in sharp contrast to the response to TRF 2 activity. Thus, the TRF 1 activity can be also detected in the mice undergoing chronic GVHR. The fact that parental thymocyte-injection into F_1 mice induced TRF 1 activity may imply that the TRF 1 represents one of the previously reported allogeneic effect factors, i.e. alloantigen-stimulated T cell–derived factors.

We next addressed the question of whether or not the GVHR-induced TRF 1 is also responsible for the polyclonal B-cell stimulation and autoantibody production. The results showed that only chronic GVHR induced by the spleen cells (but not by the thymocytes transfer) resulted in a severe SLE-like syndrome characterized by the appearance of Coomb's antibody and proteinuria; these results strongly support the notion that TRF 1 is not responsible for the induction of autoantibody. In fact, the injection of B151-TRF 1 into normal mice did not induce any anti-Bm-MRBC PFC response.

It has been shown that introduction of the *xid* genetic mutation into lupus-prone mice significantly delays SLE onset and reduces polyclonal B-cell activation and autoantibody production (110–113). Unresponsiveness to TRF 2 by CBA/N *xid* mice would support the theory that even the overproduction of L-BCDF(B151-TRF 2) would not trigger the induction of autoimmunity. We, therefore, examined the B-cell responses of *xid* (CBA/N × DBA/2J)F_1 male mice and their non-*xid* counterparts of female mice. The B cells from F_1 female mice responded to the signals provided by L-BCDF and B151-TRF2, whereas in male *xid* mice B-cell responses to these B-cell differentiation factors drastically reduced the polyclonal Ig-secreting cell response and autoantibody production. These results demonstrate that introduction of the *xid* mutation does indeed render their B cells unresponsive to differentiation signals provided by TRF 2, as has been described for L-BCDF (105). These observations for B151-TRF 2 activity are in sharp contrast to the Sidman's BMF, which was reported to be able to induce differentiation of resting CBA/N B cells to the same magnitude as the B cells from normal nondefective mice (69).

It is possible to speculate that role of TRF 2 in physiologic B-cell differentiation is such that a pool of activated T cells in vivo may continuously secrete low levels of TRF 2 which, in turn, sustain Ig secretion by resting B cells. In some types of autoimmune diseases such as exemplified by MRL/lpr mice and chronic GVHR, control mechanisms that maintain normal Ig levels may be aberrantly affected by the rapid expansion of the TRF 2–producing T-cell population.

SIGNIFICANCE OF CLASS-II MHC-MEDIATED T-B CELL INTERACTION FOR INDUCTION OF B-CELL DIFFERENTIATION FACTOR FROM T CELLS AND ACQUISITION OF B-CELL RESPONSIVENESS TO THE FACTOR

We have described several physiologically distinct B-cell differentiation factors and discussed certain aspects of receptor-ligand interactions for the expression of their biological activities. However, it is not known whether a subpopulation of B cells, or all of the B cells, eventually require such factors for their differentiation, since in T-B cell interactions, helper T cells may produce several soluble factors stimulatory to the resting as well as activated B cells, in addition to participating directly in the MHC-restricted cell-cell interaction. Isolation and characterization of these factors that govern the B-cell differentiation are valuable for analyzing certain aspects of cellular and molecular events in the differentiation process of B cells as discussed above. However, the interpretation of these phenomena should be made cautiously: It is not known whether a series of factors can act on a single B cell. Alternatively, interactions of various cell populations during MHC-mediated or idiotypic immune circuits and networks may be differentially sensitive to the effect of these molecules. The following lines of evidence will illustrate that the acquisition of B-cell responsiveness to the differentiation factors is indeed tightly connected to the class-II MHC-mediated cell-cell interactions, and they will provide another intriguing complexity of the factor-mediated B-cell differentiation mechanism (Figure 3).

Ia-Bearing B Cells as Stimulator Cells for the Factor Production of T Cells

After the demonstration that B cells also bear Ia antigens (114) and that at least some interactions between antigen-specific helper T cells and B lymphocytes are Ia restricted (5, 6, 13), many investigators have attempted to determine whether B lymphocytes can also function as antigen-presenting cells. In fact, B cells present antigens to T cells and could stimulate immune T lymphocytes to proliferate in an Ia-restricted fashion (115–123). Convincing evidence for the role of B cells in presenting antigen came from studies of Chesnut & Grey (115), who showed that macrophage-depleted splenocytes cultured with rabbit antimouse immunoglobulin (Ig), but not rabbit IgG lacking anti-Ig activity, could stimulate rabbit IgG-primed T cells to proliferate in vitro. This result not only established the ability of B lymphocytes to present protein antigens to T cells, it also

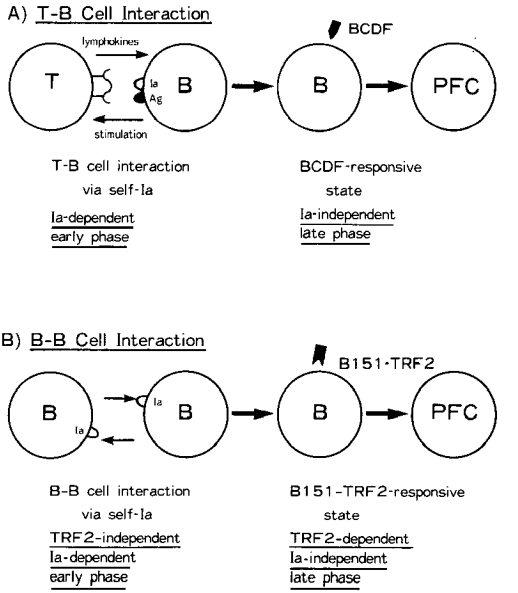

Figure 3 (A) Ia-mediated T-B-cell amplification loop for induction of B-cell differentiation factor from T cells and acquisition of B-cell responsiveness to the factor. (B) Ia-mediated B-B cell interaction for determination of responsivity of B cells to the B151-TRF 2. PFC, plaque (antibody)-forming cell.

indicated that their surface Ig receptors played an important role in this phenomenon. Subsequently, it has been shown that resting and mitogen-activated normal B lymphocytes, as well as a variety of Ia-positive B cell–derived tumors and cell lines, are capable of presenting foreign proteins to Ia-restricted, antigen-reactive T lymphocytes (116–123). Rock et al (124) also demonstrated that hapten-specific B lymphocytes are highly efficient at presenting hapten-protein to T cells for producing IL-2, and that hapten-binding surface Ig molecules are critically involved in this effective form of T-B interaction. Moreover, in their system it appeared that the major role of surface Ig is to concentrate or "focus" antigen onto the relevant B cells. Other studies have found that Ig-receptor may capture antigen. Subsequently, the processed antigen is expressed on the cell surface in the context of Ia (125). These results raised the possibility that antigen-binding B cells may serve an important role as antigen-presenting cells in physiologic immune responses. The ability of B cells to present nominal protein antigens to inducer T cells has several implications for physiologic immune responses. It may provide an accessory cell–independent mechanism for stimulating inducer cells to secrete helper factors that are necessary for clonal expansion and differentiation of B lymphocytes. The critical role

of surface Ig in this phenomenon suggests that B cells are most efficient at presenting the antigen for which they express specific membrane receptors. This provides an explanation for the original observation that physiologic H2–restricted cognate T-B collaboration occurs in vivo (5, 6) and in secondary antibody responses to low-dose antigen challenge in vitro (126).

Ia-Mediated Signal Transduction by T Cells for Acquisition of B-Cell Responsivity to the Factor

Looking at the B-cell side, binding of antigen (or anti-Ig) to the B-cell Ig receptor has the role of rendering that cell sensitive to a battery of differentiation factors including B151-TRF 1 that effectively amplify the specific response to that antigen (41, 42, 44, 45). In addition, researchers have reported the existence of lymphokines including BSFp1 that induce an increase in the expression of Ia antigen on B cells (40, 127). Therefore, in the T-B cell interactions, T cells may be activated by B cells, and then activated T cells provide signals through production of various lymphokines for the activation of B cells. Some lymphokines from activated T cells as well as the antigen binding to Ig receptors induce an increased expression of Ia molecules on B cells, and these exert a positive feedback effect on the cell-cell interactions. This process may represent a signal transmission mechanism provided by direct interactions of H-2 restricted T and B cells, and the B-cell activation signals may also be transduced through Ia molecules on B cells. This can be also deduced by the following observation made by Kimoto et al (128): Nonactivated *xid* B cells from CBA/N mice or (CBA/N × C57BL/6)F_1 male mice do not respond to BSFp1 and/or BCDF for proliferation and Ig secretion. However, when *xid* B cells were cocultured with an H 2–restricted helper-T-cell clone for 24 hr, proliferation and Ig secretion could be induced in those cells with BSFp1 and BCDF, showing that direct interaction with a helper-T-cell clone activates *xid* B cells to the stage responsive to BSFp1 and/or BCDF. These results are highly consistent with our observation (82–86) that CBA/N B cells have TRFs receptor and that, once stimulated by the Ia-mediated pathway including antigen-priming in vivo, they become responsive to B151-TRF 1.

ROLE OF CLASS-II MHC-MEDIATED B-B CELL INTERACTION FOR DETERMINATION OF RESPONSIVITY OF B CELLS TO THE B151-TRF 2

Another intriguing role of Ia-mediated cell-cell interaction for determination of responsivity of B cells to the B-cell differentiation factor has emerged from the B151-TRF 2 response analysis. The B151-TRF 2 directly activates

small resting B cells in the complete absence of any T-cell effect. However, in this reaction, class-II MHC-mediated B-B cell interaction was found to play an obligatory role in the determination of TRF 2 responsivity of B cells.

Ia-Mediated B-B Cell Interaction for Determination of B151-TRF 2 Responsivity

A crucial role of Ia-mediated B-B cell interaction for acquisition of TRF 2 responsivity was demonstrated by the drastic inhibition of TRF 2 response when monoclonal anti-Ia antibody was added to the culture. The inhibition of anti-Ia antibody was observed only in B151-TRF 2–induced PFC response, but not in B151-TRF 1–induced response. The class-I MHC-specific antibody did not inhibit both responses. It is possible that inhibition of TRF 2–induced PFC response by anti-Ia antibody is due to blocking of binding of TRF 2 to the TRF 2 receptor by virtue of a plausible steric hindrance effect of antibody. But this possibility is negated by the fact that the ability of B cells to absorb TRF 2 activity was not changed after the treatment of B cells with anti-Ia antibody.

Further detailed kinetic studies of the inhibition of anti-Ia antibody in TRF 2 response demonstrated that inhibition of anti-Ia antibody was effective only when it was added at the initiation of the culture, and delayed addition (on day 2) of antibody did not inhibit the 4- or 5-day culture of the TRF 2–induced PFC response. In a series of these kinetic studies, it was also found that the stimulation of B151-TRF 2 on day 2 induced almost the same magnitude of PFC response as the day-0 stimulation, while TRF 2 stimulation given for the initial 2-day culture, in which the cells were then washed off, did not induce any substantial response. This suggests that some cellular activation process precedes the time when the resting B cells become responsive to TRF 2 and that this process is the one inhibited by the anti-Ia antibody. Thus, the process of B-cell differentiation induced by TRF 2 can be divided into two steps: The first step is Ia-mediated B-cell activation (thus, an anti-Ia antibody–inhibitable, but TRF 2–independent) process which proceeds within 2 days after the culture. The second step commences around 2 days after the culture is now in the Ia-independent but TRF 2–dependent phase. These two steps in the distinct process of B-cell activation in response to TRF 2 are schematically depicted in Figure 3.

In order to study more stringently the existence of an Ia-mediated B-cell activation process in response to the TRF 2, additional experiments were carried out using (P1 × P2)F_1 B cells, and inhibitory activities of anti-Ia antibodies with either one of the parental specificity were examined. Interestingly, the magnitude of inhibition of TRF 2 response of F_1 B cells evidenced by either one of the antiparental Ia antibody was approximately

half that of the parental B cells, suggesting that F_1 B cells may be separated into two populations that recognize specifically the respective parental Ia. This is reminiscent of the response of MHC restricted F_1 T cells (129). To substantiate this point, F_1 B cells were separated into adherent and nonadherent cell population by panning onto either one of the parental monolayer B cells, and the specificity and magnitude of the inhibition of anti-Ia antibody was examined.

The results unequivocally demonstrated that the TRF 2 response of (P1 × P2)F_1 B cells adherent to the P1 or P2 B-cell monolayer was selectively inhibited by anti-P1 or anti-P2 Ia antibody, but not by irrelevant reciprocal combination of anti-P2 or anti-P1 Ia antibody (S. Ono, Y. Takahama, T. Hamaoka, manuscript in preparation). These findings strongly suggest that (P1 × P2)F_1 B cells comprise at least two populations with selective binding capacity to either one of the parental Ia-positive B cells and that the activation of resting B cells to the TRF 2–responsive state requires B-cell recognition of B-cell class-II MHC product. Thus, these studies employing selective cell separation technique and specific anti-Ia antibody-induced inhibition of TRF 2 responses of F_1 B cells strongly support the notion that B cells recognize polymorphic class-II MHC product for response to B151-TRF-2 (Figure 4).

While each of the studies noted above attempted to control or account for the self Ia–recognition requirements of B-B cell interaction for determination of TRF 2 responsivity of B cells, it might be argued reasonably that cryptic Ia-positive accessory-cell contamination was present in the monolayer of the B-cell populations employed in these

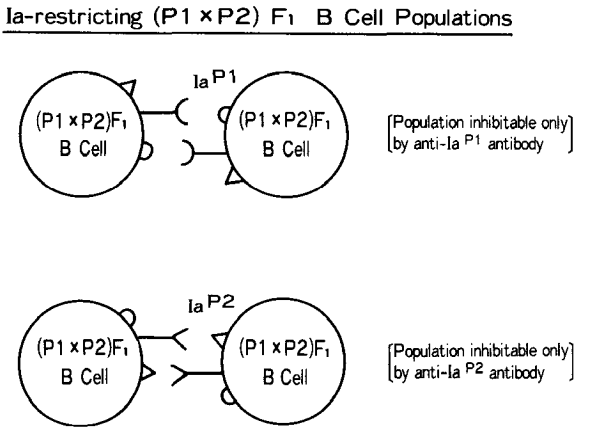

Figure 4 Haplotype-specific self-Ia restriction of (P1 × P2)F_1 B cells in B151-TRF 2–induced B-cell differentiation.

studies and that all of the observed self Ia–recognition requirements were in fact accessory cell–mediated, Ia-restricted B-cell response. However, one set of experiments was carried out in which this possibility was excluded though the use of anti-Ig-purified B cells, and it was found that the Ig-positive B-cell monolayer is also effective for the self Ia–specific separation. In these separation experiments, it was also found that pretreatment of responder B cells with anti-Ia or anti-Ig antibodies did not affect the separation efficiency, whereas treatment of monolayer B cells with anti-Ia antibody completely abolished the Ia haplotype–specific separation of responder B cells. Moreover, the effective separation of B cells was also accomplished by H-2 identical, but background disparate, B-cell monolayer, and I-A subregion identity between monolayer cells and responder B cells was required (Y. Takahama, S. Ono, T. Hamaoka, manuscript in preparation). These findings are also consistent with the notion that TRF 2–responsive B cells are capable of discriminating the class-II MHC determinants expressed by B cells.

Self-Ia Recognizing Structure on B Cells

A number of recent studies have now suggested that B cells as well as T cells may express specificity for self-MHC determinants as well as for nominal antigens. We previously reported that B cells obtained from BALB/c mice that had been immunized with M104E myeloma protein have an idiotype-specific enhancing activity for antidextran B1355S antibody responses in vivo. The enhancing cells were Thy-1$^-$, Lyt-1$^-$, 2$^-$, nylon wool adherent, and rabbit anti-mouse Ig-dish adherent (130). The significance of these B-B cell interactions was further analyzed in vitro by Yamamoto et al (131), using various Igh-1 and H-2 congeneic strains of mice. The results demonstrated that the capability of enhancing B-cell induction is related to the producibility of the respective idiotype in that strain of mice. Moreover, successful cooperation between those two types of B cells requires the same class-II MHC haplotype.

In an analogous manner, Sherr et al (132) demonstrated that the B-B cell interaction activity is restricted by genes linked to the Igh complex, a result that probably reflected its Igh-linked NP^b-idiotype specificity. Moreover, B cells are H-2 restricted and the H-2 restriction maps to the I-A subregion. These investigators claimed that the B cells involved are Lyt-1$^+$ subpopulation.

Probably more convincingly, for the system of MHC restriction between a specific subpopulation of antigen-specific B cells and accessory cells, Singer & Hodes analyzed the response stimulated by the polysaccharide antigen TNP-Ficoll (133). Antibody responses stimulated by TNP-Ficoll require Ia-positive accessory cells and exhibit both a T-independent and a

T-dependent component. For the T-independent component of the TNP-Ficoll response (134), it was observed that spleen cells from (P1 × P2)F_1 into parent P1 and from full allogeneic (P1 into P2) radiation bone marrow chimeras were MHC-restricted in their interactions with accessory cells, so that these chimeric B cells only cooperated with host-type but not donor-type accessory cells.

If the B cells are capable of simultaneously expressing specificity for both nominal antigen or idiotype and self-Ia determinants, what is the nature of the cell-surface receptors that mediate this specificity? Antigen and self-Ia could be recognized either by a single receptor or by two distinct receptors. If recognition were mediated by a single receptor on the B cell, the antigen-specific immunoglobulin receptor may be the most apparent candidate for this function. The implication of such a hypothesis is that the cell-surface Ig receptor, and presumably its secreted antibody counterpart, would express specificity for both antigen and self-Ia. Although most antibodies have classically been identified through their ability to bind antigen alone, recent examples have been reported of antibodies that uniquely recognize nominal antigen in association with class-I MHC products (135, 136). It remains to be determined whether some Ia-restricted antibodies can be identified in significant proportion, especially for antibodies generated by the TRF2 stimulation, particularly under the condition in which B-cell activation appears to be Ia-restricted. However, IgM antibody with such self Ia–restricting specificity was not detected in the 7-day-culture supernatant in our complement-dependent cytotoxicity assay, which indeed detected murine anti-Ig allotype antibody activity to the B cells under the same condition.

Alternatively, it is possible that B-cell recognition of nominal antigen and of self-MHC are mediated through two receptors and that the receptor for self-MHC determinants is distinct from the immunoglobulin receptor for antigen. In fact, in our experiment, capping off Ig-receptor from responder (P1 × P2)F_1 B cells by anti-Ig treatment before monolayer separation did not affect the subsequent separation efficiency and specificity of responder B cells, strongly suggesting that Ig-receptor is not involved in the selective binding of F_1-responder B cells to the parental B-cell monolayers. In our opinion, it is this experimental result that is most difficult to reconcile with the concept that the Ia recognition structure of the TRF 2–responsive B cells is an immunoglobulin receptor.

Adaptive Differentiation of the Self Ia–Recognizing Structure on B Cells

Finally, we addressed the question of whether Ia-restriction specificity of TRF 2–responsive B cells can be adaptively differentiated in allogeneic

chimera. The results illustrated that the PFC responses stimulated by TRF 2 in the presence of mitomycin C–treated P2 B cells as the costimulator at the spleen cells of (P1 into P2) allogeneic radiation bone-marrow chimera mice were inhibited only by P1-specific anti-Ia antibody, but not by P2-specific anti-Ia antibody. This shows that the self Ia–restriction specificity of TRF 2–responding B cells is not adaptively differentiated in the heavily X-irradiated host environment, particularly even in the presence of radiation-resistant Ia-positive accessory cells. However, this does not exclude a possibility of adaptively differentiating properties of the self Ia–recognizing structure of B cells. If there exists any anti-self Ia-differentiating process, the possibility remains that this might occur among the transferred bone-marrow-cell population (intra-bone marrow education). In fact, this occurred when the mixed bone-marrow-cell chimera was established by transferring (P1 + P2) bone-marrow cells into heavily X-irradiated (P1 × P2)F_1 recipients: The TRF 2 response of the P1-derived cells in recipients (obtained after P2-specific anti-H-2 antibody plus complement-treatment) eventually became sensitive to the inhibition of anti-Ia antibody specific for the allogeneic P2 haplotype, when the response was induced in the presence of mitomycin C–treated P2 B cells as costimulator. Thus, the restriction specificity of the self Ia–recognizing structure on TRF 2–responsive B cells can be educated to become reactive to other Ia-haplotype specificity, when the B cells are cultured in the mixed bone marrow chimera (Figure 5) (S. Ono, Y. Takahama, T. Hamaoka, manuscript in preparation).

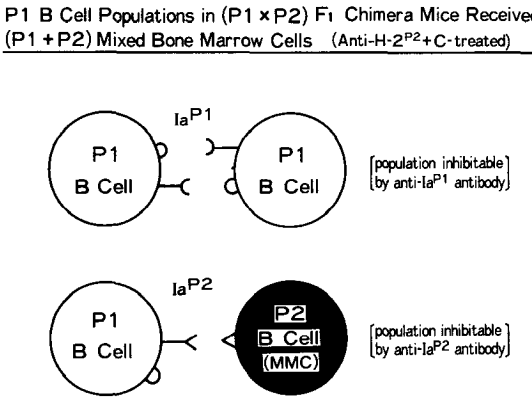

Figure 5 Adaptive differentiation of P1 B cells to become reactive to P2 allogeneic B cells in the B151-TRF 2 response. P1 B cells were prepared by anti-H-2^{P2} antibody plus C-treatment of the spleen cells from heavily X-irradiated (P1 × P2)F_1 chimera mice reconstituted with (P1 + P2) mixed bone marrow cells. It was established by other experiments that mitomycin C-treated (MMC) P2 B cells alone do not respond to TRF 2 to generate PFC but possess Ia-restricting stimulatory activity to the MMC-nontreated B cells to respond to the B151-TRF 2.

Implication of Self Ia–Restricted B-B Cell Interaction

The need for recognition of self-Ia determinants by B cells in response to B151-TRF 2 increases enormously the complexities of lymphokine-receptor interactions. Indeed, it raises the provocative possibility that each Ia-restricted cell-cell interaction involved in the activation of B cells might simultaneously involve a number of discrete sets of receptor-ligand interactions in which each partner cell expresses Ia determinants as well as anti-Ia receptors and TRF 2 receptor as well. The current widely held immunological concept has affirmed that MHC-restricted self recognition is a property unique to T cells. The experimental findings reviewed here represent a significant challenge to the contemporary prediction or assumption that the T-cell-receptor molecule is a unique self MHC–recognizing element. It challenges also the concept that lymphokines perform a simple task as differentiation-inducers to a single B-cell mediated by rather simple receptor-ligand interactions. A more definitive analysis of this issue may await the availability of systems that will allow the assessment of B-B cell interactions on a clonal level and activation requirements for individual B cells or the isolation of Ia-recognizing receptors from putatively Ia-restricted B-cell populations. Further exploration of the mechanisms of self Ia–mediated B-B cell interaction for excitation of B cells by TRF 2 may provide us with new insights into the class-II MHC-centered lymphocyte-activation process, as well as an intriguing feature of the self MHC–recognizing element of B cells, identical (or analogous) to, or distinct from, that of T cells.

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CONTENTS

Ann. Rev. Immunol. 1986. 4 : 205–30

THE PANSPECIFIC HEMOPOIETIN OF ACTIVATED T LYMPHOCYTES (INTERLEUKIN-3)

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INTRODUCTION

The T-cell lymphokine that forms the subject of this review has been studied using a variety of bioassays, under many different names. As the discussion below indicates it is now apparent that burst-promoting activity, CFUs-stimulating activity, histamine-producing cell stimulating factor, interleukin-3, mast-cell growth factor, multicolony-stimulating factor, P cell–stimulating factor, and WEHI-3 factor are all products of a single gene. The diversity of these assay systems reflects the fact that this single lymphokine stimulates hemopoietic cells from every major hemopoietic lineage. Although there is some controversy, no well-substantiated evidence indicates that this lymphokine acts on lymphoid cells. Because this lymphokine clearly functions as a hemopoietin with a characteristically broad range of targets, where a general term is required the factor will be described as panspecific hemopoietin (PSH).

HISTORY

In 1963, the first report of the growth in vitro of cells of hemopoietic origin documented the generation of mast cells from cultures of thymic cells (1). Because the cells used in these experiments were taken from noninbred mice, the generation of these mast cells very likely depended on the release of PSH from T cells activated by allogeneic interactions.

The myelomonocytic leukemia WEHI-3B that has played a central role

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in the molecular and biological analysis of PSH was reported six years later (2). Tissues from WEHI 3–bearing mice released an activity that stimulated colony formation in agar cultures of normal bone marrow (2). This colony-stimulating activity probably reflected the secretion of PSH; a decade later, descendants of WEHI-3B were used as sources of burst-promoting activity (3), interleukin-3 (IL-3) (4), mast-cell growth factor (5, 6), P cell–stimulating factor (7), and WEHI-3B factor (8). Moreover, the constitutive production of PSH by this myelomonocytic leukemia led to the notion that aberrant production of PSH by a hemopoietic cell could lead to autostimulation and thus might be a critical step in leukemogenesis (9).

In 1974 Cerny (10) reported that the T-cell mitogen phytohemagglutinin enhanced the survival in vitro of pluripotential hemopoietic stem cells (CFUs). This was the first indication that activated T cells secreted a molecule that stimulated pluripotential hemopoietic stem cells. Some five years later Schrader et al showed that CFUs-stimulating activity (CFUs-SA) was directly synthesized by the activated T cell (11, 12) and, in subsequent work, that CFUs-SA was identical with PSH (13). At present PSH appears to be the only hemopoietin able to stimulate the in vitro production of pluripotential hemopoietic stem cells.

The capacity of medium from cultures containing activated T-cells to stimulate the growth of colonies of megakaryocytes (14) may in hindsight be accounted for by the action of PSH as a megakaryocyte colony-stimulating factor. Likewise PSH can account for the capacity of medium conditioned by activated T lymphocytes to stimulate the growth not only of large colonies of erythroid cells, originally termed burst-promoting activity (3) or erythroid colony-stimulating factor (15), but also colonies of multiple lineages of hemopoietic cells including erythroid cells, megakaryocytes, neutrophils, and macrophages (15, 16) an activity initially termed mixed-colony stimulating factor. Burgess et al characterized in broad molecular terms the colony-stimulating activities present in medium conditioned by activated T cells (17) but were unable to come to definitive conclusions about the number of molecular species responsible for the various activities.

ASSAYS FOR PSH

The characterization of this molecule has depended largely on two major assays. The first depended on the unique ability of PSH to support the in vitro growth of factor-dependent mast cells or persisting (P) cells (5, 11, 18–22) or of certain factor-dependent lines of other hemopoietic lineages (8, 23). The specificity and simplicity of assays based on the stimulation of mast-cell growth and survival led to their widespread use in the characterization and purification of the molecule (7) and ultimately to the isolation of the cDNA clones that now effectively define the molecule (24, 25).

The second assay that played a major role in the characterization of this factor was that for interleukin-3 (26). This involved the induction of an enzyme 20-α-hydroxysteroid dehydrogenase (20-α-SDH) in cultures of the spleen cells from congenitally athymic (mu/mu) mice. Although the IL-3 assay did not find widespread use, largely because of the relative technical complexity of the assay of 20-α-SDH, its importance lay in the fact that Ihle and colleagues used it to purify IL-3 and obtain the first information about the N-terminal amino acid sequence of the protein (4).

Assays Using Factor-Dependent Lines

The finding that lines of murine mast cells could be maintained in vitro using a soluble factor released from activated T cells was made independently by a number of workers. Schrader (18, 19, 27, 28) showed that homogeneous populations of cells that shared many characteristics of mast cells could be reproducibly obtained by culturing cells from normal lymphohemopoietic tissues together with medium conditioned by concanavalin A–activated spleen cells or T-cell hybridomas. These cells were initially termed persisting (P) cells, in reference to their characteristic persistence in these cultures after other cell types had disappeared. P cells could be cloned but were not immortal, and subsequent work has established that they represent the in vitro counterparts of a normal subset of mast cells. The subset has been variously termed the "atypical," "thymus-dependent," or "mucosal" subset of mast cells (29).

Hasthorpe (20), also in Melbourne, independently reported a permanent line of mast cells that grew from cultures of cells from the spleen of a mouse infected with the Friend murine-leukemia virus. The line was also dependent upon a factor released by mitogen-activated spleen cells. Tertian et al (30), Razin et al (21), and Nabel et al (22) all independently reported the growth of mast cells from normal tissues using medium that had been conditioned by activated spleen cells or T cells, while Nagao et al (6) reported that similar cell lines were derived from long-term bone marrow cultures using as a stimulus medium conditioned by the myelomonocytic leukemia WEHI-3B.

WEHI-3B conditioned medium had also been used by Greenberger et al (31) and Dexter et al (32) to obtain permanent factor-dependent cell-lines from virus-infected (31) or normal (32) long-term bone marrow cultures. These immortalized lines had the characteristics of early myeloid cells. Dexter's group later used the growth of these lines to characterize PSH.

PERMANENT VERSUS NONPERMANENT FACTOR-DEPENDENT LINES Permanent, factor-dependent lines corresponding to many types of hemopoietic cells have been reported. These include mast cells (20) and early myeloid cells (31, 32)—bipotential cells giving rise to megakaryocytes

and mast cells (33), or megakaryocytes and erythrocytes (34), and even multipotential lines giving rise to neutrophils, macrophages, eosinophils, megakaryocytes and erythroid cells, etc (35). These permanent factor-dependent lines corresponding to a variety of hemopoietic lineages differ from the factor-dependent mast cells that persist in culture for only limited periods in several important respects.

One key distinction lies in the likelihood of the presence of genetic abnormalities in the permanent lines. The prolonged but not indefinite, PSH-dependent growth of normal mast cells in tissue culture appears to reflect a physiological capacity for a limited amount of self-renewal in well-differentiated murine mast cells of this subset. In contrast, the capacity for indefinite self-renewal demonstrated by the permanent lines probably results from genetic abnormalities (see 33). This notion is supported by the fact that whereas the nonpermanent lines of factor-dependent mast cells can be obtained quite reproducibly from normal tissues, the permanent lines of factor-dependent hemopoietic cells occur only infrequently and unpredictably. Moreover the frequency with which immortalized lines of hemopoietic cells can be established is increased by infection of the cultures with murine leukemia viruses (31), or by the use of donor mice that have high levels of endogenous murine leukemia viruses (36) or have been infected with murine leukemia viruses (37). Prolonged passage in vitro of the permanent lines also may select for karyotypic abnormalities (33).

Specificity of the Factor-Dependent Cell-Line Assays for PSH

Earlier results indicated that the effect of PSH on the growth of lymphokine-dependent mast cells was specific and could not be replaced by lymphokines such as the granulocyte-macrophage colony-stimulating factor (38, 39). interleukin-2 (5), or interferon-γ (39), or by other hemopoietins such as macrophage colony-stimulating factor (27). These results do not of course exclude the possibility that these factors could have effects on mast cells apart from the stimulation of growth. For example, interferon-γ has been shown directly to influence the expression of major histocompatibility antigens on PSH-dependent mast cells (40). At present, PSH appears to be the only factor capable of stimulating the growth of P cells or lymphokine-dependent mast cells. Certainly, it is clear that the three different groups that used the mast-cell growth-factor assay to purify a protein (7) or to isolate cDNA clones (24, 25) were all studying the same molecule; this suggests that, at least in the cases of the sources they used, the mast-cell growth assay probably detects a single product.

The question of whether assays based on the stimulation of growth of murine–mast cell lines can be used to define unequivocally the presence of

PSH is an important one. For example, assays based on permanent factor-dependent murine–mast cell lines have yielded positive results with soluble factors from human sources (41) and from nonlymphoid murine tissues, e.g. keratinocytes (42) or astroglia (43). However, to establish unequivocally that a mast-cell growth-stimulating molecule from a novel source is PSH or a related molecule, additional data are necessary. These could include evidence of competition between the factor and labeled-PSH for binding to the PSH receptor, reactivity with antibodies specific for defined regions of PSH, or the presence in the cellular source of mRNA that hybridizes with PSH cDNA probes.

The need for additional tools in the characterization of factors stimulating lines of hemopoietic cells is emphasized by reports that permanent PSH-dependent cell lines can in some instances respond to multiple lymphokines (44, 45). The instance of factor-dependent lines of myeloid progenitor cells that respond to both PSH and GM-CSF (45) should not be surprising, as normal myeloid progenitor cells have receptors for not only PSH but also GM-CSF (33). When used in parallel with a target line that responds only to PSH, these lines form a convenient assay system for GM-CSF. Another myeloid line, the monocytic leukemia WEHI-274.14 responds to three hemopoietins—PSH, GM-CSF, and macrophage-CSF (CSF-1) (J. W. Schrader & S. Schrader, unpublished results), a pattern of responsiveness that again resembles that of the normal counterpart of the line, in this case a macrophage progenitor.

More intriguing are reports that permanent mast-cell lines can respond to IL-2 (44). Although this may reflect a genetic aberration that has occurred in a permanent cell line, this observation should provoke a search for a possible role of IL-2 in mast-cell physiology. It is a provocative fact that the various hemopoietic growth factors and IL-2, while showing no obvious structural interrelationships, can nevertheless interchangeably promote the growth and survival of certain cell lines. This fact implies that the interaction of these various factors with their respective receptors results in the activation of common pathways and opens up the possibility that pathological growth of hemopoietic cells could result from the aberrant expression of lymphokine receptors not normally characteristic of these cells.

Assay of Interleukin-3

The enzyme 20-α-hydroxysteroid dehydrogenase is assayed by its ability to metabolize radiolabeled progesterone (26). This enzyme attracted Ihle's attention as a potential marker of T-cell differentiation because it was expressed in medullary thymocytes but not cortical thymocytes. Moreover, it was absent in the spleen of congenically athymic (*nu/nu*) mice but could

be rapidly induced by a factor present in medium conditioned by mitogen-stimulated spleen cells (26). Because it was assumed that the induction of 20-α-SDH reflected stimulation of the differentiation of T-cell precursors, and because he had shown that the factor was distinct from interleukin-2, Ihle suggested that this third putative factor in T-cell differentiation should be termed interleukin-3 (26).

It is now clear that the notion that this assay is measuring a parameter of T-cell differentiation is incorrect. There is no direct evidence that IL-3 has any effect on 20-α-SDH levels in any T-cell or committed T-cell progenitor. Moreover the enzyme 20-α-SDH is not specific for the T-cell lineage and is expressed by myeloid cells (46–48), including mast cells (49). The observed induction of 20-α-SDH by PSH (IL-3) can be readily accounted for by the well-documented effects of PSH on hemopoietic progenitors of lineages known to express 20-α-SDH. Moreover, it has been suggested that the induction of 20-α-SDH is not unique to PSH. Thus Hapel et al (48), although not Ihle et al (50), have observed that a distinct hemopoietin, GM-CSF, also induced an increase in the levels of 20-α-SDH in hemopoietic tissues. Hapel et al also observed increases in 20-α-SDH with a third cytokine, macrophage-CSF or CSF-1, and concluded that the presence of 20-α-SDH was characteristic of proliferating hemopoietic cells of certain lineages, regardless of the hemopoietin with which they were stimulated.

MOLECULAR CHARACTERIZATION OF PSH

The first evidence that the factor stimulating the growth of mast-cell lines was a direct product of T cells came from the demonstration that it was released by Con-A stimulation of cloned T-cell hybridomas (18, 27). Subsequently, Yung et al reported that the factor was released by mitogen-activation of a T-cell lymphoma, LBRM (5), and Nabel et al showed that it was produced by activation of long-term clones of helper T lymphocytes (22). Ihle showed that IL-3 was produced by mitogen activation of the T-cell lymphoma EL4 (49). Later, limit-dilution strategies were used to demonstrate that short-term T-cell clones produce the spectrum of bioactivities now known to be characteristic of PSH (51).

One exception to the apparently exclusively T-cell origin of PSH was the production of a factor that stimulated the growth of permanent lines of myeloid cells (31, 32) or mast cells (5, 6, 52) by the myelomonocytic leukemia WEHI-3B. Likewise, Ihle and colleagues observed that WEHI-3B was a good source of interleukin-3 (53). Because the WEHI-3B lines that were studied by these workers expressed high levels of the Thy-1 antigen (54), there was some speculation that WEHI-3B represented an abnormal T cell.

However, as we indicate below, this conclusion was not valid because normal myeloid progenitors express the Thy-1 antigen when stimulated by PSH (55), and the expression of the Thy-1 antigen by a myelomonocytic leukemia may merely reflect a freezing of differentiation of the neoplastic cell at the stage of an activated myeloid progenitor cell.

Early studies established that PSH was a heavily glycosylated protein and that glycosylation accounted for most of the charge-heterogeneity of the protein (56). The apparent M_r on gel filtration ranged from 45,000 (22) to 30,000 (56). By gel-filtration under dissociating conditions in the presence of 6M guanidine-hydrochloride, the mean M_r was estimated at 23,000 (56). By SDS-gel electrophoresis PSH was heterogeneous with a mean M_r of about 25,000 (7, 8).

Initial studies seemed to indicate that IL-3 as assayed by the induction of 20-α-SDH differed significantly from the molecules studied in the assays based on the growth of mast cells or factor-dependent hemopoietic lines. Thus, purified IL-3 was reported to have an apparent M_r on SDS gel-electrophoresis of around 40,000 (49). Subsequently, low molecular weight forms of IL-3 were reported (57); the apparent M_r of the dominant form was 28,000, with a smaller amount of material with M_r of 32,500. The high M_r form of IL-3 initially reported seems to represent a heavily glycosylated species, as peptide maps suggest that the protein is identical with low M_r IL-3 (58).

Initial biochemical and biological studies pointed to strong similarities between the mast-cell growth factor and IL-3 made by activated T cells and those made by WEHI-3B. Because WEHI-3B produced relatively high levels of bioactivity constitutively, without the need for stimulation by mitogens, it was a convenient source of material for the attempts at the purification and detailed molecular analysis of the molecule which, although extremely potent, was produced in very small amounts.

The severe logistic problems involved in obtaining sufficient amounts of purified material for analysis of the amino acid sequence were first solved by Ihle et al who used 150 liter batches of WEHI 3B–conditioned medium (57). Using the induction of 20-α-SDH as an assay, Ihle et al obtained sufficient, homogeneous material for limited amino acid sequencing and reported a unique N-terminal amino acid sequence (4). Ihle sent purified IL-3 to a number of laboratories that had been studying factor-dependent mast cells or other factor-dependent lines, and these collaborative studies demonstrated that purified IL-3 was active in these multiple systems (57).

Clark-Lewis et al subsequently reported the purification of PSF (7) again from WEHI-3B conditioned medium. This material had a unique N-terminal amino acid sequence that differed from that of IL-3 in that there

were an additional six N-terminal amino acid residues, after which the sequence coincided with that of IL-3 (7). The possible significance of this difference is discussed below.

Recombinant DNA Studies

The sensitivity and specificity of the mast-cell growth factor assay made it relatively simple to detect PSH production by Xenopus oocytes that had been injected with fractionated mRNA from WEHI-3B or activated T cells (59). However, the low abundance of the PSH mRNA made isolation of the appropriate cDNA clones a daunting task, and a number of strategies were tried by the various interested groups. In the event, two laboratories succeeded in isolating virtually identical cDNA clones, both using the translation of mast-cell growth-stimulating activity from hybrid-selected mRNA in Xenopus oocytes as a screening strategy. Young's group in Australia (Fung, 24) isolated a cDNA clone from WEHI-3B, as did Arai's group in California (Yokota, 25) from a helper T-cell clone developed by Nabel. The sequences of the two clones differed by only a single base, pointing to the identity of the factor produced by the myelomonocytic leukemia WEHI-3B and the T-cell line.

Arai's group subsequently isolated the gene and showed that it contained five exons (60). Examination of the noncoding sequences suggested the presence of an enhancer-like structure in the second intron and a TATA-like sequence preceded by a G + C rich region in the 5′-flanking region of the gene. Comparative analyses of the molecular mechanisms involved in the induction of this gene and of the genes coding for other T-cell lymphomas, e.g. IL-2, interferon-γ, and GM-CSF, are underway in a number of laboratories and may lead to fundamental insights into the regulation of mammalian genes.

Structure of PSH

A relatively clear picture of the primary structure of PSH has now emerged. The nucleotide sequence of the full-length cDNA clones that have been isolated predict that the primary translation product is a polypeptide of 166 amino acids. Data on the N-terminal amino acid sequence of PSF (7) indicate that mature PSH is formed by cleavage of this 166–amino acid polypeptide between positions 26 and 27 at a glycine-alanine bond, a site likely to be susceptible to cleavage by enzymes involved in the processing of secreted proteins. The further cleavage of the six N-terminal acids required to generate the IL-3 sequence reported by Ihle et al (4) would then occur at an arginine-aspartic acid bond, a potential site for attack by proteolytic enzymes. Both the 140–amino acid (PSF) and the 134–amino acid (IL-3)

forms are heavily glycosylated (56, 58), accounting for the apparent M_r of about 25,000 on SDS-PAGE (7, 8, 57).

Affinity-purified antibodies against synthetic peptides offer one approach to the question of which form of PSH is the major natural product. Experiments using affinity columns made with affinity-purified antibodies specific for residues 1–6 of PSH have demonstrated that at least 80% of the bioactivity in medium conditioned by either activated T cells or WEHI-3B or in the serum of mice bearing WEHI-3B tumors has the six additional amino acids present on PSF but not IL-3 (H. J. Ziltener, I. Clark-Lewis, B. Fazekas, S. B. H. Kent, L. E. Hood, J. W. Schrader, in preparation). Analysis of PSH produced by the activated T-cell lymphoma, LBRM, indicates that, like PSF, it has the six N-terminal amino acids not found on IL-3 (61).

Data obtained by many groups using IL-3 prepared by Ihle suggest that the initial 6 N-terminal amino acids of PSH are not critical for any of its various biological activities (4). The possibility that these results could be due to low-level contamination of the 7–140 form (IL-3) by the 1–140 form (PSF) has been excluded by experiments in which the 7–140 form has been synthesized nonbiologically and shown to have all of the bioactivities of PSH (61a). Nevertheless, further experiments are required to determine whether there are subtle differences between the two forms of PSH in terms of their bioactivity or in vivo behavior. Certainly, available data indicate that the 140–amino acid species of PSH is the major product both from the physiological source (the activated T cell) and from pathological sources (e.g. myeloid leukemias such as WEHI-3B) and is likely to be the most significant species in vivo.

Chemically Synthesized Analogues of PSH

The demonstration that biologically active polypeptides corresponding to the entire 140 amino-acids of PSH could be synthesized using automated peptide synthesis (61a), has led to a new and rapid approach to analysis of the structures critical for bioactivity. Clark-Lewis et al (61a) have synthesized analogues that have demonstrated the importance for bioactivity of the cysteine residue at position 17. Similar experiments have shown that the first 16 amino acids are not essential for bioactivity and that a peptide corresponding to residues 1–79 at the N-terminal half of the molecule has detectable biological activity (61a).

It seems from these data that a loop stabilized by a disulfide band between the cysteine at position 17 and the cysteine at either position 79 or 80 is important for biological activity. The cysteine at position 140 probably forms a disulfide bond with the unpaired cysteine at either position 79 or 80, thus creating a second loop. These details should be

rapidly elucidated by ongoing work, which, together with analyses of monoclonal antibodies that neutralize the bioactivity of PSH (H. J. Ziltener, I. Clark-Lewis, B. Fazekas, S. B. H. Kent, L. E. Hood, J. W. Schrader, unpublished), should clarify the nature of the active site.

BIOACTIVITIES OF PSH

Definitive conclusions about the bioactivity of PSH are possible now that the purified native protein (4, 7), material expressed from molecularly cloned cDNA (62), and finally chemically synthesized polypeptides (61a) are available. Knowledge of the targets of PSH is now limited not by uncertainties about the chemical homogeneity of the biologically active material but solely by the biology of the assay systems.

Action on Hemopoietic Progenitor Cells and Differentiated Cells

The clearest conclusions about which cells interact directly with PSH come from systems involving homogeneous populations of target cells or single, isolated target cells. The use of a cloned population of mast cells has established that pure IL-3 (4), pure PSF (7), products of expression of cDNA clones (62), and chemically synthesized PSH (61a) directly stimulate the growth of mast-cell lines. Single isolated hemopoietic progenitor cells have been shown to respond to pure PSF by generating neutrophils, macrophages, megakaryocytes, and mast cells (13); burst-promoting activity purified from WEHI-3B has been shown to stimulate single, isolated progenitor cells to form erythroid bursts or colonies containing mixtures of erythroid and myeloid cells (63). Thus, there is now rigorous evidence that PSH directly stimulates the progenitors of multiple lineages of hemopoietic progenitor cells.

It is important to note that the action of PSH is not limited to immature hemopoietic cells. Purified PSH stimulates the division of well-differentiated mast cells (13) and also regulates their expression of major histocompatibility complex antigens, antagonizing the action of interferon-γ (40). Furthermore, experiments in which pure PSH has been added to single, isolated peritoneal macrophages have demonstrated that PSH directly stimulates cell division and phagocytosis in well-differentiated macrophages (64).

Another broad category of experiments has dealt with the effect of PSH on heterogeneous populations of cells. Such experiments do not allow definitive conclusions about whether PSH directly interacts with a given cell but do demonstrate important effects on the hemopoietic system as a whole. When added to suspensions of bone-marrow cells in medium gelled

by agar, purified PSH stimulates the growth of colonies of megakaryocytes, mast cells, neutrophils, and macrophages (59, 13). Burst-promoting activity (BPA) was purified using the stimulation of colonies (bursts) of erythroid cells or of mixtures of erythroid and myeloid cells (3, 63). Precisely the same effects are seen with material generated by the expression of cloned cDNA (62) or with synthetic PSH, produced by nonbiological peptide synthesis (61a)

The known bioactivities of PSH clearly can account for many of the hemopoietic cell-stimulating activities released by activated T cells; these activities include megakaryocyte colony–stimulating factor (14), burst-promoting activity (63), and mixed colony–stimulating factor (15). Although PSH acts as a granulocyte macrophage colony–stimulating factor in stimulating the growth of colonies of neutrophils and macrophages (59), the majority of the granulocyte macrophage colony–stimulating activity released by activated T cells is due to a distinct and unrelated molecule (38, 65), usually termed granulocyte-macrophage CSF (GM-CSF) or sometimes CSF-2 (see Ref. 33 for a review of the factors regulating hemopoiesis). Material expressed from cloned cDNA also generates colonies of eosinophils in agar cultures of bone marrow (62), indicating that the eosinophil colony–stimulating activity in T cell–conditioned medium is contributed to by PSH. However, GM-CSF also has some eosinophil colony–stimulating activity, particularly in the human (66), and there may be additional eosinophil-stimulating factors released by T cells (33).

Although, at present, PSH appears to be the only factor that alone stimulates the formation of colonies of mast cells, megakaryocytes, or mixed colonies, this situation could change. It is worth noting that at least four discrete hemopoietins–namely, granulocyte macrophage colony–stimulating factor; granulocyte colony–stimulating factor; macrophage colony–stimulating factor; and PSH—stimulate to some degree the progenitors of neutrophils and macrophages (33). Likewise, erythropoiesis may be influenced by at least two hemopoietins in addition to PSH, namely, erythropoietin and a T-cell lymphokine, erythroid-potentiating activity (67).

Thy-1-Inducing Activity

The first evidence that the expression of the Thy-1 antigen on myeloid cells was regulated by a T-cell lymphokine came from experiments in which T cell–depleted bone-marrow cells were cultured together with medium conditioned by activated T cells. By 3 days, large numbers of cells expressing high levels of the Thy-1 antigen had appeared (55). The relevant factor was produced by activated T-cell hybridomas and by WEHI-3B and had the same apparent M_r as PSF (55). Separation of the Thy-1 positive

cells by fluorescence-activated cell sorting demonstrated that they included the progenitors of mast cells, neutrophils, macrophages, and also pluripotential hemopoietic stem cells capable of forming macroscopic colonies of erythroid and myeloid cells in vivo. No evidence emerged that the Thy 1–positive population contained cells committed to the T-cell lineage; although the Thy-1 positive cells were large blasts, they did not survive or grow in the presence of IL-2. These data indicated that expression of Thy-1 could no longer be regarded as unequivocal evidence of commitment to the T-cell lineage amongst these cells.

Later experiments have shown that purified PSF (59, 13), purified IL-3 (58), products of expression of cDNA clones (62), and synthetic PSH of nonbiological origin (61a) are all active in inducing the expression of the Thy-1 antigen on bone-marrow cells. Very low levels of Thy-1 antigen have been detected on normal hemopoietic progenitor cells that had not been deliberately stimulated (68); this suggests that PSH increases a preexisting low level of expression of Thy-1 antigen. This increase is transient, as mature hemopoietic cells including mast cells are Thy-1 negative in the mouse. The expression of high levels of Thy-1 antigen on activated myeloid progenitor cells fits with the presence of Thy-1 antigen on factor-dependent myeloid lines (32) and the myelomonocytic leukemia WEHI-3B (54).

Action on Pluripotential Hemopoietic Stem Cells

Early studies indicated that the T-cell factor that stimulated mast-cell growth (PSF) copurified with a factor, CFUs-stimulating activity (CFUs-SA), that supported the in vitro culture of pluripotential hemopoietic stem cells (12). The latter were identified as CFUs (colony-forming units—spleen), i.e. as cells that when injected into lethally irradiated mice were able to give rise to macroscopic colonies of erythroid and myeloid cells. Experiments using purified PSF (13, 59), purified IL-3 (J. N. Ihle & J. W. Schrader, unpublished), material expressed by cloned cDNA (62, J. W. Schrader & A. J. Hapel, unpublished), and chemically synthesized PSH (61a) have conclusively established that CFUs-SA is identical with PSH.

This action of PSH involves stimulation of division of CFUs or their precursors and not merely stimulation of their survival, as PSH increases the proportion of CFUs that can be killed using cell cycle–specific cytotoxic agents (13). The pluripotential hemopoietic stem cells that are stimulated by PSH and give rise to colonies present one week after injection into irradiated mice may represent stem cells that have a limited capacity for self-renewal (see 33). However, PSH also supports the culture of stem cells that presumably have a significant self-renewal capacity in that they give rise to splenic colonies present as late as three weeks after injection into

congenitally anaemic W^+/W^+ mice (J. W. Schrader, unpublished data). To demonstrate that the effect of PSH on pluripotential hemopoietic stem cells with high self-renewal capacity is a direct one will require experiments that are technically very demanding. However, PSH has been shown to directly stimulate isolated pluripotential cells capable of giving rise to colonies containing multiple hemopoietic lineages in vitro (63).

In Vitro Culture of Cells Giving Rise to B Lymphocytes and Thymocytes

Purified PSH directly or indirectly supports the in vitro culture of cells that are capable of giving rise to thymocytes and T and B lymphocytes when injected into irradiated animals (13; J. W. Schrader and J. Clark-Lewis, in preparation). Bone-marrow cells were cultured for 6–7 days in the presence or absence of pure PSH and then injected into irradiated hosts. The use of donors and hosts with different allelic forms of the Thy-1 antigen permitted identification of thymocytes and T cells derived from donor bone-marrow cells that had been cultured with the PSH. In experiments aimed at identifying cells that gave rise to B lymphocytes, the donor bone marrow was from CBA or CBA.T6.T6 mice, and the hosts were of the CBA/N genotype and thus lacked B lymphocytes that were capable of forming colonies in agar. Repopulation of the B-lymphocyte compartment by donor cells from PSH-stimulated cultures was established by the demonstration of B-lymphocyte colony-forming cells in the reconstituted mice and by the presence of the T6 chromosomal-marker in B-cell blasts isolated from the reconstituted animals.

These experiments do not establish that PSH directly affects any cell that has become committed to the lymphoid differentiation pathway. The results are just as consistent with the notion that PSH stimulates a pluripotential lymphohemopoietic stem cell that only give rise to cells of lymphoid lineage after transfer into the host animal. Moreover, there is at present no direct evidence that this effect of PSH is a direct one, as the target population of bone-marrow cells is heterogeneous and consists of many cell types. Nevertheless, this action of PSH may form a useful practical tool in the analysis of lymphopoiesis.

DOES PSH OR IL-3 ACT ON T CELLS?

The fact that the induction of 20-α-SDH by PSH that formed the basis of the IL-3 assay (26) can be accounted for by the well-documented stimulatory effect of PSH on hemopoietic progenitor cells does not necessarily exclude the original notion that IL-3 also stimulates the

induction of 20-α-SDH in T-cell precursors. However, at present there is no good evidence that IL-3 induces 20-α-SDH in T cells or their precursors or indeed has any direct effect whatsoever on these cells.

One piece of evidence in favor of the notion that IL-3 acted on T cells or their precursors was the claim that purified IL-3 promoted the growth of clones of helper T cells that expressed Lyt.1 and Thy-1 antigens (69). These lines secreted IL-3 constitutively and could be induced by tetradecanoyl-phorbol-acetate to secrete IL-2 (69). However, these data have not been reproducible even in the original laboratory (49, 58). One explanation for some of these data is that the putative helper-T-cell lines were in fact WEHI-3B cells that had contaminated the cultures, as WEHI-3B cells express the Thy-1 antigen and secrete IL-3 constitutively.

Palacios has reported a somewhat similar line of experimentation, indicating that purified IL-3 or medium conditioned by WEHI-3B stimulates the growth of lines of T cells derived from human peripheral blood cells (70). These lines eventually become independent of exogenous growth factors, and Palacios suggested that the growth of these variants depended upon the autogenous production of an IL-3-like factor. No other laboratory, however, has reported similar findings.

Ihle has reported that Thy 1–positive, IL 3–dependent lymphoma lines can be derived from mice infected with Moloney murine leukemia virus (58). However, as noted above, the Thy-1 antigen is no longer acceptable as an unequivocal marker of membership of the T-cell lineage in the mouse, as it also occurs on activated myeloid progenitors (55). Before accepting that IL-3 dependent cells or cells derived from an IL-3 dependent line belong to the T-cell lineage, it will be important to have evidence that other T cell–specific properties, e.g. rearrangement of T-cell-receptor genes or expression of the T-cell receptor, are present.

Ihle also reported that IL-3 was necessary for the establishment in vitro of IL-3-independent lines that lacked IL-3 receptors from mice with primary tumors induced by murine Moloney leukemia virus (58). Ihle has suggested that the IL-3-independent lines had arisen in vitro from IL-3-responsive cells that had differentiated to a stage at which they no longer responded to IL-3. However, an alternative possibility is that the IL-3-independent cells were present in the original inoculum from donor animal and that consequently the effect of IL-3 on the establishment of these cells in vitro was an indirect one involving the stimulation of PSH-responsive cells such as macrophages or mast cells. Certainly, careful analysis of experiments of this nature using unambiguous markers of cell lineage and genetic markers of clonality, e.g. the presence of unique sites where viral DNA is integrated, is important because it is possible that some of the PSH-dependent lines from mice infected with murine leukemia virus may turn

out to be counterparts of early cells in the hemopoietic or lymphoid differentiation pathways. As yet, however, there are no well-documented reports of PSH-dependent cell lines capable of generating T lymphocytes.

Thymocytes or T-cells do not have detectable receptors for PSF (71) or IL-3 (72, 58). Nor do thymocytes respond in any detectable way to either molecule (58, J. W. Schrader, unpublished data). There are reports that IL-3 can affect T-cell responses in vitro, for example, enhancing the generation of cytotoxic-T cells (73) or suppressor-T cells (74). However, before accepting the notion of a direct interaction of PSH with T cells, it is important to exclude an alternative explanation based on the well-substantiated effects of PSH on hemopoietic cells. Thus, the ability of pure PSH to stimulate mature macrophages (64) or regulate the expression of Ia antigens on mast cells (40) points to the possibility that PSH could influence immune responses by stimulating the production or function of accessory cells of hemopoietic origin (75).

PSH and Nonspecific Cytotoxicity

The question of enhancement of cell-mediated cytotoxicity by PSH is complicated by the fact that nonlymphoid cells that are known to respond to PSH, including mast cells and monocytes, have cytotoxic activity (reviewed in 33). PSH enhances natural cytotoxic activity in cultures in vitro (76) and supports the growth of lines that have NC-activity (77), although the lineage of the cells stimulated is not clear. However, at present there is no evidence that the effect of PSH on cell-mediated cytotoxicity involves a direct effect on cells of the T-cell lineage.

DOES PSH DIRECTLY AFFECT PRE-B OR B LYMPHOCYTES?

The only evidence that PSH directly affects cells committed to the B lymphoid pathway comes from Palacios and colleagues (76). These workers reported that lines of PSH-dependent pre-B cells could be reproducibly isolated from fetal liver. In most cases the identification of these lines as pre-B lymphocytes rested on their expression of an antigen detected by a monoclonal antibody. However, it has yet to be shown that this antigen is specific for pre-B cells and is absent from from cells of other hemopoietic lineages such as mast cells.

In the case of one of the IL-3-dependent "pre-B-lymphocyte" lines, evidence was presented of the presence of rearrangements of the immunoglobulin genes typical of the B-lymphocyte lineage (76). The data on this cell-line appear unambiguous but, as yet, similar lines have not been reported by other laboratories. If PSH-dependent pre-B lymphocytes can

be obtained as readily and reproducibly as this paper claims, it is difficult to understand why these results have not been readily reproduced. Certainly, if confirmed these experiments represent an important milestone in the in vitro study of lymphopoiesis.

Pike & Nossal (76a) observed no effect of pure PSH on the growth or differentiation of a single antigen-stimulated B lymphocyte, and the failure of spleen cells to absorb detectable amounts of PSH (71) suggests that mature B cells (and T cells) lack receptors for PSH. As we indicated above, while PSH permits the in vitro culture of cells capable of giving rise to B lymphocytes in irradiated hosts, there is no evidence that these cells are committed to the lymphoid lineage. At present the work of Palacios (76, 77) stands alone as the only evidence of direct effects of PSH on B lymphopoiesis.

MECHANISM OF ACTION OF PSH

Evidence for a Cell-Surface Receptor

The initial interaction of PSH with its target cells occurs via a specific cell-surface receptor. The first evidence for such a receptor came from experiments demonstrating that contact of PSH-dependent cells with PSH at 4°C, resulted in the specific absorption of biological activity (9, 71). Specific absorption was also observed when lightly fixed cells were used, and biologically active PSH could be eluted from these fixed cells using glycine buffer at pH 2. With the bioassay it was possible to quantitate accurately in units of biological activity the amount of PSH bound and the affinity of PSH for the receptor (71). Assuming a specific activity of PSH that corresponds to a 50% maximal level of stimulation in the PSH bioassays at a concentration of pure PSH of 1.3×10^{-13} M, these experiments indicate that PSH-dependent lines had about 1000 available PSH receptors per cell and that these receptors interacted with PSH with Kd of approximately 5×10^{-12} M.

Palaszynski and colleagues have used radiolabelled pure IL-3 and have characterized cell-surface receptors on a number of cell types (72). The numbers of available receptors on PSH-dependent lines were in approximate agreement with those determined by absorption, although the Kd differed—at about 5×10^{-11} M.

Palacios & Garland (77) were unable to demonstrate the absorption of PSH by PSH-dependent mast cells, although they were able to demonstrate the absorption of PSH by a PSH-dependent cell that they identified as a pre-B lymphocyte. It is difficult to explain the marked discrepancy between their results and those of Crapper et al, which clearly demonstrated absorption by mast cells (9, 71), although the conditions of growth of PSH-

dependent cell lines do have quantitative effects on absorption capacity, and down-regulation of the receptor can occur (unpublished data). Certainly, Palaszynski et al (72) have demonstrated the specific binding of radiolabelled IL-3 to mast-cell lines. At present, it seems unlikely that Palacios & Garland (77) are correct in proposing that PSH stimulates mast cells by a mechanism that does not involve a cell-surface receptor.

Experiments in which PSH-dependent cell lines have been incubated with PSH at 37°C have demonstrated that metabolically active PSH-dependent cells consumed PSH at a high rate (R. M. Crapper, J. W. Schrader, in preparation). Up to a concentration of PSH that was more than 10-fold higher than that necessary for the maximal stimulation of growth, the rate of consumption increased with increasing concentrations of PSH. Consumption of PSH at 37°C could be blocked by metabolic inhibitors such as sodium azide, and also by chloroquine (K. Leslie, J. W. Schrader, unpublished data), suggesting that the consumption of PSH involved internalization and recycling of receptors. In vivo, consumption of PSH by PSH-dependent cells may be a significant mechanism for controlling local levels of PSH.

Antibodies to the PSH Receptor

Evidence that PSH acts via a cell-surface receptor also came from experiments with antisera produced by immunizing rabbits with PSH-dependent cells and extensively absorbing these sera with mouse tissues that lacked the PSH receptor (78). Antibodies prepared from these sera were able to mimic the effects of PSH and stimulated both PSH-dependent cell-lines and normal hemopoietic progenitor cells. Antibodies to the PSH receptor should be useful in purifying the PSH-receptor and analyzing the effects of blocking the action of PSH in various pathological conditions.

Metabolic Effects of PSH

Dexter and colleagues have suggested that a critical effect of PSH on target cells is an elevation of intracellular ATP and that PSH can be substituted for, at least to a limited extent, by an ATP-generating system (79). After contact of PSH-responsive cells with PSH, one early event is enhanced glucose uptake (80). Farrar et al have also reported that PSH induces a redistribution of C-kinase from the cytoplasm to the membrane (81).

Physiology of PSH

PSH was discovered and characterized in in vitro systems. Subsequent in vivo experiments have now established that the physiological role of PSH conforms largely with that predictable from in vitro observations. PSH is released in vivo when T-cells are activated by antigen (82–85). The effects of

this PSH are usually local and are restricted to the vicinity of the activated T cells, i.e. the site of immunization or sites to which activated T cells migrate (82). It is only with severe immunological stimuli, such as parasitic infestation (84) or graft-versus-host disease (85), that PSH occurs in the serum. PSH is not complexed to carrier proteins in the blood (85). It is filtered by the kidney, and in pathological conditions where it enters the serum, PS is detectable in the urine, although the relative levels in serum and the urine suggest that it is degraded to a significant extent in the kidney. The half-life of PSH in the blood is relatively short—of the order of 40 minutes (83, 86).

The effects of the release of PSH differ at different anatomical sites depending on the availability of particular targets cells. In some locations such as the gut mucosa, the most prevelant target is a cell that is committed to the generation of mast cells (87); T cell–dependent immune responses in the intestinal mucosa thus result in the release of PSH and stimulate a local mastocytosis. In lymph nodes the most obvious effect of the local release of PSH is again a local increase in the number of mast cells and their progenitors (82). However, at sites where other hemopoietic progenitor cells are available as potential targets, e.g. the bone-marrow or the spleen, this apparent specificity of PSH for mast cells is not seen. In these sites the release of PSH stimulates the production not only of mast cells but also of metamyelocytes, megakaryocytes, etc (83), together with cells that respond to PSH—witness the dramatic increase in IL-3 responsive cells demonstrated in the spleens of mice undergoing a chronic immune response against a murine leukemia virus (88).

One significant insight into the in vivo role of PSH came from the observation that in vitro–derived PSH-dependent mast cells that had been transferred into the skin of mice survived for a prolonged period only if the mice were carrying a PSH-producing tumor (89). If the mast cells that increase in number at the sites of immunological reactions in humans also require maintenance of local levels of PSH to stay alive, measures based on interruption of the production of PSH or its interaction with mast cells might form the basis of new therapeutic approaches to allergic disease.

These experiments indicate that the physiological role of PSH is to function as a link between the immune system and the hemopoietic system that provides many of the accessory and auxillary cells necessary for an efficient defense and repair response. There is no evidence that PSH, or at least PSH produced by T cells, plays any role in the steady-state production of any hemopoietic cells. PSH is not detectable in the serum of normal mice (83); moreover, the long-term bone-marrow cultures that generate pluripotential hemopoietic stem cells and progenitor cells do not contain detectable levels of PSH (28).

In Vivo Effect of Injected PSH

The first formal demonstration that the injection of PSH leads to increases in vivo in mast cells and other hemopoietic cells and their progenitors has come from the use of chemically synthesized PSH (61a). Treatment of mice with an 8-hourly subcutaneous injection of approximately 10^4 units of PSH for 4 days, resulted in 100-fold or greater increases in the numbers of progenitors of mast cells and other myeloid cells in the spleen (J. W. Schrader, I. Clark-Lewis, H. Ziltener, S. B. H. Kent, and L. E. Hood). Mast cells also increased in number in the spleen, gut, and in the skin at the injection site. The synthetic PSH had a half-life in the blood similar to natural PSH. These experiments point to the potential for the therapeutic use of synthetic PSH or analogues in enhancing the production and function of cells of hemopoietic origin, e.g. during bone-marrow engraftment.

ROLE OF PSH IN ONCOGENESIS

The aberrant expression of the PSH gene in cells that are normally the targets for this factor could play a key role in oncogenesis. The initial clues to this notion lay in the production of PSH by the myelomonocytic leukemia WEHI-3B. The release of PSH by WEHI-3B differed from that by T cells in that it was constitutive rather than inducible and was not accompanied by the secretion of other T-cell lymphokines such as IL-2, GM-CSF or interferon-γ. These considerations led to the hypothesis that the secretion of PSH by WEHI-3B was a reflection of the abnormal activation of a PSH gene, and that autostimulation by autogenous PSH had played a key role in the genesis of this leukemia (9, 59). The expression of the Thy-1 antigen by WEHI-3B (54) was also consistent with the notion that this myelomonocytic leukemia was the malignant counterpart of a neutrophil-macrophage progenitor cell activated by PSH.

Although studies performed on the initial isolates of WEHI-3B suggested that WEHI-3B both responded to and produced a hemopoietin (2), most clones of WEHI-3B that were in common use a decade later showed no signs of receptors for or responses to PSH. This situation may reflect their long period of culture in vitro, or alternatively, the very high levels of PSH that they produce—as Whitton et al (80) did detect a response of WEHI-3B to PSH at 20°C, a temperature at which the secretion of PSH was reduced.

Southern blot analysis of a number of clones of WEHI-3B, including those that had been used for the isolation of PSH and others that were independently isolated, demonstrated that one copy of the PSH gene had been rearranged (60; K. Leslie, J. W. Schrader, S. Schrader, in preparation).

The rearranged gene has recently been cloned and shown to be constitutively expressed as a result of the insertion of an intracisternal-A particle into the 5′ flanking sequence (8a). These results clearly support the notion that secretion of PSH by WEHI-3B is pathological and, in the light of evidence discussed below, was likely to have had a direct bearing on the pathogenesis of this tumor.

Evidence that Activation of the PSH Gene is an Oncogenic Change

Direct evidence that abnormal activation of the PSH gene could be a critical event in leukemogenesis came from in vitro experiments with variants of a PSH-dependent line (9). In these studies 50 million PSH-dependent cells of an immortalized, mast-cell/megakaryocyte line were plated in agar in the absence of an exogeneous source of PSH. A small number of autonomous colonies subsequently grew and were recloned in the absence of exogeneous PSH. These autonomous variants were producing a factor with the biological properties of PSH; moreover, their rate of growth in vitro was strongly dependent on the concentration of PSH in the culture medium or upon the cell density in the cultures. These two observations suggested that the growth of the cells still depended on the external interaction of PSH with cell-surface receptors. Furthermore, unlike the parental line, these autostimulatory variants were leukemogenic and gave rise to disseminated mast-cell/megakaryocyte leukemias when they were injected into syngeneic animals.

Further experiments using Northern blots have demonstrated that activation of the PSH gene has occurred in the variant line, since PSH mRNA of normal size are present in the leukemogenic variants but not the parental line (90). These results were supported by biochemical and immunological studies that have demonstrated that the molecule secreted by these leukemic variants has molecular characteristics identical with PSH (90) and reacts with antibodies specific for peptides corresponding to parts of the amino acid sequence of PSH (H. J. Ziltener, I. Clarke-Lewis, K. Leslie, S. B. Kent, L. E. Hood, and J. W. Schrader). Experiments with independently derived variants of the same line also demonstrated the coincident onset of the autonomous production of PSH and of leukemogenic behavior in vivo. These observations established conclusively that the initiation of the aberrant production of PSH can be a critical step in oncogenesis.

Aberrant Activation of PSH Gene in Leukemias Arising in Vivo

Further evidence that the abnormal activation of the PSH gene played a role in myeloid leukemias that had arisen in vivo came from observations

on the monocytic leukemia WEHI-274, which arose in a mouse that had been infected with Abelson-murine-leukemia virus. A cloned line, WEHI-274.14, was isolated from an early passage of the tumor and was shown both to produce PSF and to require it for in vitro growth (90).

Southern-blot analysis of DNA from WEHI-274.14 showed that one PSH gene had been rearranged. Moreover, Northern-blot analysis indicated that these cells produced a polyadenylated RNA transcript of grossly abnormal size (K. Leslie, J. W. Schrader, S. Schrader, in preparation). Biochemical characterization (90) and immunological analyses using antibodies to peptides corresponding to defined parts of PSH (H. J. Ziltener, K. Leslie, S. B. H. Kent, L. E. Hood, and J. W. Schrader) have demonstrated that the PSH-like molecule secreted by WEHI-274.14 is indistinguishable from T cell–derived PSH.

The experiments on the in vitro genesis of leukemogenic lines by activation of the PSH gene (9) demonstrated that aberrant activation of the PSH gene can be a leukemogenic step. Taken together with the evidence discussed here that the PSH gene is abnormally rearranged in two myeloid tumors that arose in vivo, i.e. WEHI-3B and WEHI-274, these results suggest that autostimulation by PSH constitutes one important mechanism of leukemogenesis in cells of hemopoietic origin. Interruption of autostimulation using either antibodies against PSH or the PSH-receptor, or synthetic antagonists of PSH, may form the basis for novel and relatively specific and nontoxic therapies for diseases involving this mechanism. For this reason it will be important to determine whether a similar mechanism is involved in any neoplastic disease involving the hemopoietic system in the human (discussed in 91).

SUMMARY

The combined deployment of cellular, biochemical, and recombinant DNA techniques has resulted in the emergence of a relatively clear picture of the molecular structure of PSH and of a number of other T-cell lymphokines. Already there is evidence that new techniques, in particular, the chemical synthesis of biologically active peptides, will lead rapidly to a detailed picture of the secondary and tertiary structure of PSH and its active sites. Moreover, the availability of antibodies to the receptor and the use of recombinant DNA techniques should result in the molecular characterization of the cell-surface receptor and eventually a detailed structural analysis of its interaction with PSH. Given the likelihood that PSH may play a pivotal role in chronic allergic diseases and possibly in hematological disorders, there are real prospects that analogues and antagonists of PSH may provide a basis for new therapeutic approaches to these diseases.

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Annual Review of Immunology
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CONTENTS

Ann. Rev. Immunol. 1986. 4 : 231–51

MOLECULAR IMMUNOBIOLOGY OF COMPLEMENT BIOSYNTHESIS: A Model of Single-Cell Control of Effector-Inhibitor Balance

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INTRODUCTION

The twenty proteins of the complement system are synthesized in liver, but several are also produced at extrahepatic sites. Blood monocytes, tissue macrophages, epithelial cells of the gastrointestinal and genitourinary system, and fibroblasts are among the extrahepatic cells shown to synthesize complement in tissue culture. Complement synthesis and its regulation has also been examined in cell lines derived from hepatomas and myelomonocytic tumors. The functional importance of an extrahepatic source of complement has been established only for mononuclear phagocytes. These cells synthesize the complement effector and control proteins [except for the terminal components of the membrane attack complex (C6, 7, 8, 9)]. In fact, the macrophage-derived complement proteins are produced in sufficient amounts to opsonize particles for complement receptor–mediated phagocytosis without addition of plasma (1). Activation products of the complement proteins produced locally alter vascular permeability and smooth muscle tone, and direct migration of leukocytes. Furthermore, these proteins participate in recruitment of intravascular humoral and cellular constituents that enhance and then regulate inflammation.

The net biological effects of the regulation of complement gene expression must be understood in the context of local concentrations of the rate-

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limiting activation proteins and inhibitory proteins of the complement cascade. Hence, changes in transcription, posttranscriptional processing, translation, and posttranslational modification for each of the activating and inhibitory proteins must be assessed. Moreover, interactions of complement proteins with other products of the macrophage, including several enzymatic cascades and enzyme inhibitors, introduce an additional level of complexity to the analysis. That is, one must also consider the molecular regulation of activators of coagulation, clot lysis, components of the collagen-collagenase system, elastase-alpha$_1$-antitrypsin system, and the oxidative burst in the analysis of the biological effects of complement proteins produced by macrophages. Finally products of the macrophage that act at remote sites (e.g. the effects of IL-1 on hepatocytes) regulate complement and other acute-phase protein concentrations in plasma and therefore influence the later phases of inflammation and tissue repair. This last level of control reveals one of the several selective and independent mechanisms regulating expression of the complement genes in hepatocytes and macrophages (Figure 1).

THE COMPLEMENT CASCADE

Two distinct pathways initiate the complement activation sequence. The classical pathway is triggered by interaction of the first component of complement (C1) with antibodies of the IgG and IgM classes in complex

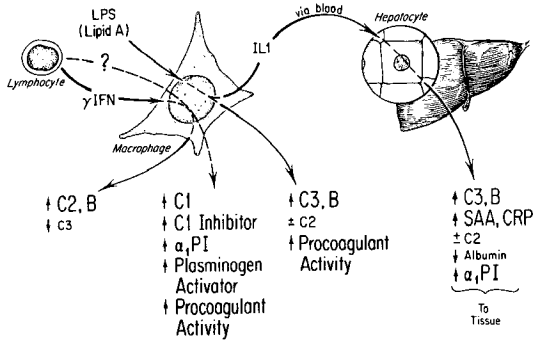

Figure 1 Expression of complement proteins by human mononuclear phagocytes and hepatocytes. Local and remote factors regulate expression of complement proteins at each site and also regulate other products of mononuclear phagocytes and hepatocytes. Products of mononuclear phagocytes (e.g. IL-1) may also regulate expression of the products of hepatocytes.

with antigen. C1 is a macromolecule consisting of three noncovalently bound subcomponents—C1q, C1r, and C1s. The C1q subcomponent is a 460 kd (kilodalton) protein composed of 18 polypeptides in 6 collagen-like triple helical units (A, B, and C chains), each terminating in carboxy-terminal globular regions and serving as the immunoglobulin-binding domains. The collagen-like regions bind dimers of the C1r and C1s subcomponents, each in its zymogen-form (reviewed in 2, 3). On cell surfaces, a single IgM or IgG dimer complexed with antigen binds and activates a single C1 molecule (4). Activation is the result of cleavage of the single-chain C1r protein (90 kd) generating an amino-terminal 60 kd peptide disulfide linked to a 30 kd carboxy-terminal fragment. The latter bears the active enzymatic site for C1r cleavage. The active C1r enzyme then cleaves zymogen C1s to activate this subcomponent by conversion of the single-chain protein to a disulfide-linked heterodimer (5, 6). C1s and C1r are structurally and functionally homologous proteins.

The natural substrates of the C1s enzyme are the fourth (C4) and second (C2) components of complement. The fourth component is a three-chain disulfide-linked glycoprotein ($\sim$200 kd), and C2 is a single-chain glycoprotein of ($\sim$102 kd). A 10 kd amino-terminal peptide (C4a) is cleaved from the alpha chain of C4 to generate C4b. The C4b binds to antigen or antibody via covalent linkage of amino or carboxyl groups to reactive groups in C4 protein generated by scission of a thiolester bond within the C4 alpha chain (7). The two human C4 genes encode distinct proteins, one of which, C4A, reacts more rapidly with amino groups and the other, C4B, with carboxyl groups. The bound C4b complexes with the carboxyl terminal (60 kd) C2a fragment liberated from C2 by the action of C1s (8). The C4b2a complex is an unstable enzyme that facilitates cleavage, at the Arg_{77}-Ser_{78} bond, of a 9 kd peptide (C3a) from the alpha chain of the third component (C3), leaving C3b. The C3 protein, like C4 and $alpha_2$ macroglobulin, has an internal thiolester site (9) that upon activation forms covalent bonds with proteins or carbohydrates. Oxidation of C2 stabilizes the human C3-cleaving enzyme, C4b2a (10, 11). This represents one of the mechanisms that influence effective tissue concentrations of complement-activation products.

The alternative pathway enzyme (C3bBb) cleaves C3 alpha chain at precisely the same site (12) as that cleaved by the classical pathway enzyme. The Bb portion of the enzyme is the carboxy terminal 68 kd polypeptide liberated from factor B (a protein structurally and functionally similar to C2) by the action of factor D, a serine proteinase analogous to C1s. A product of the reaction (C3 $\rightarrow$ C3b) is a component of the alternative-pathway C3-cleaving enzyme. Hence, a positive feedback amplification

loop is generated during activation of this pathway. Access of control proteins to the enzyme C3bBb determines its rate of decay-dissociation and thus the extent to which this pathway participates in complement activation.

The C3a fragment of C3 is homologous to the corresponding alpha-chain-cleavage products of C4 (C4a) and C5 (C5a) (reviewed in 13). C3a has vasoactive properties and may also have immunoregulatory functions. The balance of the protein C3b and cleavage products derived from C3b are important ligands in the interaction of receptors on lymphocytes, macrophages, polymorphonuclear leukocytes, and other cells with microorganisms, soluble immune complexes, and particles to which C3b has bound covalently (14, 15). In addition, the C3b component forms a portion of the enzyme responsible for terminal complement activation and the assembly of the membrane attack complex. This complex consists of a fragment of C5 (C5b) and the sixth, seventh, eighth, and ninth components of complement. A diagram of the complement activation cascade is shown in Figure 2.

In addition to the proteins forming the activation mechanism, a number of regulatory proteins that control the complement system have been recognized. These include the C1 inhibitor, an alpha globulin that inhibits the action of the C1s enzyme, and the C3b/C4b inactivator (factor I), which in the presence of cofactors BIH (factor H) or C4 binding protein (C4BP) mediates cleavage of C3b and C4b respectively. Several membrane proteins [e.g. C3b receptor and decay accelerating factor (DAF)] also have the capacity to modulate complement activation.

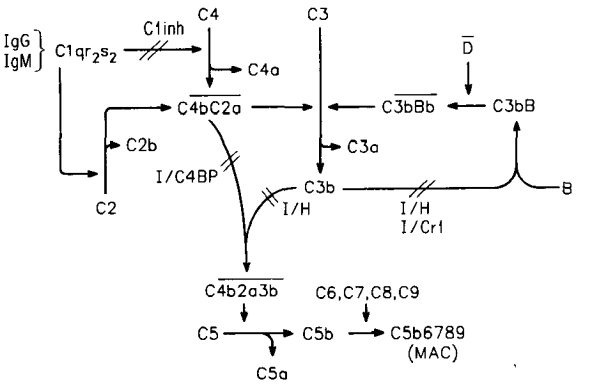

Figure 2 Complement activation pathway. Regulation of the pathway by C1 inhibitor (C1 inh), factor I/C4 binding protein (I/C4BP), factor I/factor H (I/H) and factor I/Crl receptor (I/Crl) is indicated.

ACTIVATORS OF THE CLASSICAL AND ALTERNATIVE PATHWAYS

The First Component (C1q, C1r and C1s) and Its Control Protein (C1 Inhibitor)

Biosynthesis of the C1 macromolecular complex has been demonstrated in primary and long-term epithelial cell cultures, in mononuclear phagocytes, and fibroblasts (16). In addition, hepatoma-derived cell lines (17) synthesize the subcomponents C1r and C1s. These studies indicate that the regulation of C1q synthesis is independent of the control of C1r and C1s synthesis (18), but the mechanisms have not yet been defined. The human C1q B chain is encoded by a 2.6-Kb gene that has been assigned to chromosome 1p (19). The B chain is programmed by a ~1.5-Kb mRNA, but the structure of the primary translation product and the details of postsynthetic processing, except for the known hydroxylation of lysine and proline residues, have not been studied. Inhibition of postsynthetic hydroxylation selectively inhibits C1q-subcomponent secretion (20).

Recent observations from several laboratories (21, 22) suggest that monocytes synthesize the C1 inhibitor as well as C1 subcomponents. C1 inhibitor is the major inhibitor of the classical activating pathway of complement. It is a glycoprotein with an estimated molecular mass of 105 kd and total carbohydrate content of 33% (23). C1 inhibitor blocks C1 activity by forming equimolar complexes with C1r and C1s by a covalent interaction with the active-site serine of the proteases, and in the process, it removes C1r-C1s from the C1q component (24–26). Liver is the major source of plasma C1 inhibitor (17, 27–29), but little is known about regulation of its synthesis. Recent studies by Bensa et al suggest that C1 inhibitor is present in cell-culture fluid of human monocytes incubated with a crude lymphokine mixture (21), and the lymphokine mixture contains a factor that regulates C1 synthesis as well. Molecular probes are now available for C1q and C1 inhibitor, so that the mechanisms accounting for control of their expression should soon be revealed.

Factor D

Factor D is a serine proteinase (25 kd) that circulates in plasma in its active form. Early studies (30) indicated that factor D is synthesized in mononuclear phagocytes, but definitive evidence was obtained only recently (31). In the latter, Barnum & Volanakis showed synthesis in a monocytic cell line of a factor-D protein 3 kd larger than the native protein. Regulation of factor-D synthesis and the mechanism of activation of D will be of considerable

importance because this protein is the trigger for alternative complement pathway activation.

The Second Component (C2) and Factor B

The genes encoding the major histocompatibility complex (MHC) class-III proteins C2 and factor B are on the short arm of human chromosome 6 and mouse chromosome 17. The human (32, 33) and murine (34–36) MHC class-III genes have been mapped with a series of overlapping cosmid clones. These studies reveal a similarity between the species. The 3′ terminus of the C2 gene is upstream and close (for human, ~1 Kb) to the 5′ end of the factor-B gene. There is a single copy of the C2 gene per haploid, but the fine details of C2 genomic structure have not yet been published.

Preliminary data suggest many similarities between the C2 and factor-B genes. Single loci for human (32, 33, 37) (~6 Kb) and murine (34) factor-B genes have been identified. The human factor-B gene is divided into 18 exons (37). Three exons coding for regions within the Ba fragment show significant sequence homology to one another, i.e. they may be products of tandem duplication. Active site residues in the Bb fragment are encoded on separate exons. With the exception of a unique exon found in Bb, the organization of this region of the factor-B gene is similar to that observed for other serine proteinase genes.

Human liver mRNA directs synthesis of three C2 primary products when translated in vitro (38) or in Xenopus oocytes (H. R. Colten, G. G. Goldberger, R. Kay, D. Woods, unpublished). The three forms of C2 found in human hepatoma cells and in human monocyte/macrophage cultures are probably derived from differential transcription or posttranscriptional modification. Translation of the most abundant C2 mRNA (~2.8 Kb) generates an 84 kd product that is secreted within 1–2 hours. Two forms of C2 of lower molecular mass remain cell-associated. These multiple forms of C2 have also been detected in murine L cells transfected with a genomic fragment bearing the human C2 and factor-B genes (36). In the transfectants, human C2 mRNA and C2 protein are found, but the lower molecular weight C2 mRNA is present in greater amounts than in primary cell cultures or cell lines (see below).

In extrahepatic mononuclear phagocytes, the proportion of C2-producing cells and the rates of synthesis per cell vary as a function of cellular maturation and tissue origin (39). For example, the proportion of C2-producing cells increases from the point where none is detectable in marrow and 10% in blood monocytes to 45% in spleen and peritoneal cavity. Only 2.5% of lung (bronchoalveolar lavage) macrophages produce C2, but the higher rate of synthesis of C2 per bronchoalveolar cell

compensates for the lower proportion of C2-producing cells (40). Local C2 synthesis is affected during an inflammatory response at least in part by a pretranslational regulatory mechanism. That is, the net tissue concentration of C2 is augmented not only by the increase in cell number but also by increased C2 mRNA and rate of synthesis of C2 per cell (41, 42). Interferon gamma (IFN-gamma) is one of the mediators of this change in C2 expression (see below). Preliminary data (F. S. Cole, H. R. Colten, unpublished) also suggest that in activated macrophages the specific biological activity of C2 is increased possibly by oxidation-dependent stabilization of the classical pathway C3 convertase.

Synthesis of the factor-B primary translation product (83 kd) is directed by a ~2.6-Kb polyadenylated mRNA (43). The synthesis and secretion of factor B is similar to that described for C2, but important differences, including the response to cytokines that regulate B and C2 gene expression, have been observed.

Human, murine, and guinea pig factor B are synthesized in liver and at extrahepatic sites in mononuclear phagocytes (44–46). Control of factor-B synthesis is rather complex and almost certainly is exerted at multiple levels. Recent data (A. Falus, H. R. Colten, unpublished) demonstrate a genetically determined independent regulation of B and C2 in murine hepatocytes and macrophages that is similar to the genetically determined, tissue-specific control of murine C4 and Slp noted in earlier studies. Peritoneal macrophages in exudates elicited with starch or thioglycollate synthesize factor B at about the same rate as resident cells, but specific signals (e.g. LPS, IFN-gamma) can trigger rather significant changes in expression of the factor-B gene.

In human mononuclear phagocytes, a small but significant increase in C2 and factor-B mRNA is induced by the lipid-A component of endotoxin (LPS) (47). A corresponding increase in factor-B synthesis (2–3-fold) is noted, but net C2 protein synthesis is not affected by lipopolysaccharide. These findings suggest that an increase in C2-translation rate requires a second signal, or that increased catabolism of C2 protein accounts for zero net change in C2 synthesis in the presence of LPS. Endotoxin injection into an LPS-responsive (C3H/FeJ) strain, but not the LPS unresponsive strain (C3H/HeJ), induces a dose-dependent increase in factor-B-gene expression in liver as well as in lung, kidney, intestine, spleen, heart, and peritoneal macrophages (48).

Recently the purification of IL-1 to homogeneity and isolation of cDNA clones corresponding to human and murine IL-1 (49, 50) have allowed generation of well-defined reagents for examination of the effect of these polypeptides on complement gene expression. For these and studies of

IFN-gamma regulation, we have initially focused our attention on the C2/factor-B genes, but the importance of these mediators in the control of other genes has been recognized.

IL-1 regulates factor-B expression in murine hepatocytes and in a human hepatoma cell line (51, 52). In addition, when recombinant-generated purified mouse IL-1 (rIL-1) is injected into mice (C3H/HeJ), increased factor-B mRNA is observed in both hepatic and extrahepatic sites. Whether extrahepatic factor-B-gene expression (especially in peritoneal macrophages) is regulated directly by IL-1 (autocrine or paracrine regulation) or via another cytokine (e.g. IFN-gamma) is uncertain. The latter possibility might be tested in studies of extrahepatic C2 gene expression in the IL-1 injected animals, since expression of this gene is increased by IFN-gamma but not by IL-1.

In primary murine hepatocyte cultures, both recombinant-generated murine IL-1 and highly purified human IL-1 induce a dose- and time-dependent, reversible increase in expression of the factor-B gene and a decrease in albumin gene expression. This regulation is pretranslational, since the kinetics and direction of change in specific mRNA for factor B and albumin correspond to the change in synthesis of the respective proteins. The small size of the factor-B 5′ flanking region (limited by the 3′ terminus of the IL-1-unresponsive C2 gene) provides a well-defined region to probe the structural basis for the differential effect of IL-1 on factor B and the closely related gene, C2. A cosmid bearing both genes on an ~30-Kb segment of human DNA has been introduced into mouse L cells where each is constitutively expressed (36). These MHC class III–transfected cells synthesize and secrete C2 and factor-B proteins. In the transfectant, human factor-B, but not C2, gene expression is increased when the cells are incubated with murine rIL-1 or human IL-1. The response is dose dependent and reversible.

Interferon-gamma regulates expression of both the C2 and factor-B genes in primary cell culture and in the MHC class-III transfectants (53). IFN-gamma induces a dose-dependent increase in synthesis of C2 and factor-B protein (detectable first at concentrations of IFN-gamma of 50 pg/ml). Regulation is pretranslational, but the kinetics and magnitude of the response to IFN-gamma for the two proteins is different. In both primary human monocytes and in the class-III transfectants, the IFN-gamma-induced increase in factor B always exceeds the increase in C2-gene expression.

The Third (C3) and Fourth (C4) Components

Among the complement components, C3 protein is present in plasma at the highest concentration. Native C3 is a 185 kd two-chain disulfide-linked

glycoprotein, which is synthesized as a single-chain precursor. The synthesis of prepro-C3 is programmed by a ~5-Kb mRNA. Postsynthetic cleavage by signal peptidase and a plasmin-like enzyme generates native C3 (54–57). As is the case for the precursors of C4 (pro-C4) and C5 (pro-C5), beta chain is the amino-terminal segment in pro-C3. In each, beta chain is separated from alpha chain by an arginine-rich intersubunit-linking peptide; a similar linking peptide separates C4 alpha from the carboxy-terminal gamma chain. These and other structural similarities suggest that C3, C4, and C5 have evolved by gene duplication.

The murine C3 gene is approximately 24 Kb and has been localized to chromosome 17 outside of the H-2 complex. The human C3 gene is on chromosome 19 (58). Genomic clones that include a region ~1.2-Kb 5′ to the gene have been isolated (59). Nucleotide sequence data for this 5′ flanking region are relevant to studies of C3 expression by endotoxin, IL-1, and IFN-gamma, especially since some species-specific differences have already been recognized. Endotoxin, and specifically the lipid-A component of LPS, increases net C3 protein synthesis 5–30-fold in adult human mononuclear phagocytes (47). This mechanism is largely pretranslational since the concentration of C3-specific mRNA is increased at least 5-fold. The response of C3 to LPS stimulation is also developmentally regulated as a comparison of adult and neonatal monocytes indicates. C3 and factor-B gene expression are not affected by lipid A in the neonatal monocyte, while other monocyte functions (e.g. superoxide generation) are modulated by the lipid A. Preliminary experiments suggest that C3 and B expression is enhanced in the neonatal monocytes when exposed to precursors of lipid A, to proteins associated with endotoxin, and when the neonatal cells are coincubated with adult monocytes exposed to lipid A (60). Dr. F. S. Cole and colleagues are pursuing these provocative findings.

C3 gene expression in murine hepatocytes, in a human hepatoma-derived cell line, and in murine fibroblasts is increased by either purified human or recombinant-generated murine IL-1 (51, 52). The response to IFN is species specific, i.e. human IFN-gamma affects only the human cell, and murine IFN-gamma affects only the murine cell. Apart from differences in the source of the cytokine, the response of human and murine C3 genes to IFN-gamma in mononuclear phagocytes and L-cells differs. IFN-gamma has no effect or *decreases* expression of human C3, whereas IFN-gamma induces an *increase* in expression of the endogenous murine C3. These differences in C3 regulation by IFN-gamma may reflect differences in regulatory sequences within or flanking the corresponding human and murine genes. The nucleotide sequence of the 5′ flanking region of the murine C3 gene has been reported (61). Once the human C3 5′ flanking sequence is determined, a direct comparison and site-specific mutation can

be used to test whether the response to IFN-gamma is a function of a difference in gene structure or the result of a species-specific signal transduction.

In studies of regulation of C3 expression, selective changes in translation, posttranslational modification, or secretion have not been detected. Extracellular enzymatic modification of C3b, however, constitutes another level of regulation of C3 that is well characterized. Cleavage of C3b by factor I and cofactor H generates C3bi, blocking further steps in the complement-activation sequence and in the interaction of C3b with its receptor (Cr1), but the fragment C3bi can interact with another specific receptor, Cr3 (also known as Mo1 or Mac1). Factor I, or another enzyme acting at a second cleavage site, generates a fragment, C3d-g, that inhibits lymphocyte mitogenesis and leukocyte migration (13, 62). Further cleavage by plasmin or trypsin generates still another fragment, C3d, that can bind to a distinct cell-surface receptor (Cr2) (14, 15). Therefore, the net balance of C3b, proteases, and antiproteases in the extracellular fluid has an important effect on generation of the diverse biologically active C3 cleavage products.

Synthesis of the 185 kd single-chain presursor of C4, pro-C4 (63, 64), is directed by a polyadenylated mRNA of approximately 5 kb (65, 66). Post-translational modification involves proteolytic excision of two intersubunit-linking peptides, sulfation (67), modification of residues within the region of the thiolester site (68), and glycosylation of the alpha and beta subunits (69, 70). There is extracellular cleavage of a carboxy-terminal peptide from alpha chain as well (71).

In the murine and human genome, there are two C4-like genes per haploid, but a relatively high frequency of duplications, deletions, and rearrangements lead to variations in this region. Some species such as guinea pig and hamster appear to have only a single C4 gene (72). Immediately 3′ to the human and mouse C4 genes are two genes (21-OH A and 21-OH B) with sequences corresponding to a cytochrome P450 enzyme, 21-hydroxylase (73, 74). The human C4 genes differ in size (C4A, $\sim$22 kb; C4B, $\sim$16 kb). This size difference results primarily from the presence of a larger intron in the 5′ region of the C4A gene (33). The murine S1p and C4 genes are each $\sim$16 kb and are similar in exon-intron structure and nucleotide sequence at the 5′ end of the genes (75). However, they differ in sequences within the 5′ flanking regions. This is of importance in view of the differences in regulation of C4 and S1p and is in striking contrast to the extensive sequence homology within coding regions (96% nucleotide : 94% amino acid). The 5′ flanking sequence differences cannot account for all the differences in regulation of S1p and C4 because tissue-specific factors are of importance as well. For instance, S1p is expressed in hepatocytes, but not macrophages, of most $S1p^+$ inbred strains. The notable exception is found in several strains designated $H\text{-}2^w$ (64). Regulation of C4 synthesis in

hepatic parenchymal cells and mononuclear phagocytes is also under separate control. Serum levels of C4 in different mouse strains reflect hepatic synthetic rates (76) and content of C4-specific mRNA (77), whereas macrophages from high- and low-serum C4 strains synthesize similar amounts of C4 protein (77, 78).

Mouse-resident-peritoneal cells synthesize C4, but in tissue culture the rate of secretion declines within the first few hours (78, 79). This decrease is not due to limitations of the culture, because total protein synthesis remains approximately constant and factor-B synthesis increases several fold. This change in C4 biosynthesis in culture is regulated at a pretranslational level, since the content of C4 mRNA changes in parallel and there is no difference in postsynthetic processing, rate of secretion, or stability of secreted C4 (77). Elicited murine macrophages in inflammatory exudates produce considerably less C4 than resident macrophages (79). This phenomenon is also under transcriptional or posttranscriptional control, i.e. the steady state C4-mRNA levels correspond to the amount of pro-C4 synthesis in the respective cell populations.

Important differences in regulation of C4 synthesis by murine and guinea-pig macrophages have been observed. The results indicate that differences in tissue-specific regulation may be even more complicated than were appreciated from investigations of murine C4. Approximately 10% of adherent mononuclear cells derived from guinea-pig bone marrow synthesize C4. A comparable proportion of the circulating monocyte population produces hemolytically active C4, but 40–50% of macrophages derived from several different tissues yield C4-plaque-forming cells (39). These and other data indicate that C4 gene expression varies as a function of the maturation of the mononuclear phagocyte. Secretion of C4 by guinea-pig-resident macrophages is also tightly regulated. This is accomplished by a selective and specific feedback inhibition of C4 biosynthesis that is mediated by a pretranslational mechanism (80). The direct regulation of C4 production in macrophages by extracellular C4 has important consequences for regulation of an inflammatory response. That is, it provides a mechanism for controlling constitutive secretion of C4 in resting macrophages as well as a mechanism for repletion of C4 following complement consumption or diffusion from the site of inflammation. The direct control of C4 synthesis would, thus, not depend on plasma concentrations of C4.

Factor I (C3b/C4b Inactivator), Factor H, and C4 Binding Proteins

As indicated above, expression of biologically active C3 and C4 is also influenced by expression of another inhibitor of the complement-activation sequence, factor I. Factor I is a two-chain glycoprotein that cleaves the C3b

and C4b fragments. The liver is the primary source of factor I in plasma (17). In human hepatoma–derived hepatocytes, factor I is synthesized as a single-chain precursor that undergoes glycosylation and limited intracellular proteolysis to generate the two disulfide-linked subunits of the native protein (81). Biosynthesis of factor I has also been detected in primary cultures of human-peripheral-blood monocytes (30). Expression of factor I, C3, and C4 by mononuclear phagocytes presents another example of regulation of protease and protease inhibitor in the same cell. In this case, inactivation of C3, by the inhibitor factor I, requires as a cofactor, factor H or the C3b receptor CR1 (82–85), both of which are expressed in mononuclear phagocytes and liver. Cleavage fragments characteristic of the action of factors H and I on C3 alpha chain have also been identified in the cell-culture fluid of monocytes (1). The significance of protease and inactivator in this case is not entirely clear but suggests that local secretion of complement components by mononuclear phagocytes cannot lead to unregulated complement activation. C4 binding protein (C4BP) serves a function comparable to that of factor H in its action as cofactor for the cleavage of C4b. The gene encoding C4BP, factor H, and CR1 are closely linked (86). DNA clones for each have now been isolated so that studies of gene expression can be undertaken.

The Fifth (C5) Component

The mRNA specifying pro-C5 translation is similar in size to those for pro-C3 and pro-C4 (87). C5 is synthesized in hepatocytes and in extrahepatic sites (30, 88). Our understanding of the regulation of C5 biosynthesis is incomplete, but several experiments suggest that control of C5 expression in murine macrophages may involve posttranslational control mechanisms. For example, Ooi et al demonstrated that elicited peritoneal macrophages synthesized five times more immunochemically detectable C5 than did resident peritoneal macrophages, but the cell-culture fluid of the elicited cells had 4–5 times less C5 hemolytic activity (89, 90). C5 expression by murine macrophages may also be influenced by histamine, but the mechanism for this effect is uncertain (91). The recent isolation of C5-cDNA clones (87) should make it possible to study the regulation of C5 gene expression in greater detail.

Expression of Other Proteases and Their Inhibitors

Several plasminogen activators are produced by human mononuclear phagocytes. The Bb fragment of the alternative complement pathway component, factor B, can cleave and activate plasminogen (92). Production of a more specific plasminogen activator, urokinase, by human monocytes has also been demonstrated (93, 94). Both monocytes and U937 histiocytic lymphoma cells synthesize urokinase as a single-chain 55-kd inactive

zymogen. Intramolecular proteolytic cleavage activates the enzyme, and another proteolytic reaction generates an enzymatically active 33 kd urokinase. Expression of urokinase is enhanced by conconavalin A, phorbol myristate acetate, and lymphokine supernatant, but is decreased by glucocorticosteroids (95, 96). Recently, Vassalli et al have described a high-affinity cellular-binding site on monocytes and U937 cells for urokinase (97). These studies suggest that urokinase is secreted and taken up on the cell membrane or that part of the urokinase synthesized is directed to the surface of mononuclear phagocytes. In addition, an inhibitor of urokinase has recently been demonstrated in the conditioned medium of human monocytes (93). Although the production of urokinase and a urokinase-inhibitor protease-nexin has been described for human fibroblasts (98), the urokinase inhibitor in conditioned media of monocytes does not resemble protease-nexin. Expression of both plasminogen activator and inhibitor by monocytes is not counterproductive since the protease is not ordinarily activated and therefore does not complex with inhibitor. On the other hand, during inflammation urokinase is activated so that the capacity also to express an inhibitor of urokinase may prevent uncontrolled proteolysis.

In addition to the plasminogen-activating effect of complement factor Bb, there are other interactions between the fibrolytic and complement systems. Plasmin, derived from urokinase-mediated cleavage of plasminogen, can cleave C1s, factor B (99), and C3 (100), thereby activating the complement system. Interaction with other pathways will undoubtedly become clear as more is learned about the expression of these proteases.

Proteases of the coagulation pathway are also produced by human mononuclear phagocytes. The procoagulant activity of human monocytes and macrophages has been attributed to tissue factor or tissue thromboplastin. Tissue thromboplastin is a 52-kd plasma-membrane lipoprotein that binds and activates factor VIII to initiate the extrinsic coagulation pathway (101). Regulation of mononuclear phagocyte–tissue thromboplastin activity has been studied at a functional level, but relatively little is known about its structure, structural-functional correlates, or molecular characteristics. The procoagulant activity of monocytes and macrophages is ordinarily minimal but greatly enhanced in cell culture by LPS (102, 103), mitogens (104), immune complexes (105), and complement components (106, 107). It has been shown that T-lymphocyte collaboration is required for or enhances the response of mononuclear phagocytes to LPS (108–111) and immune complexes (112, 113). Using immunofluorescent flow cytometry, T lymphocytes of the inducer/helper subset were shown to be responsible for the collaboration in human monocyte procoagulant activity (114).

Regulation of procoagulant activity produced by human monocytes

provides another example of interaction between two distinct pathways, the extrinsic coagulation and the complement activating pathways. C3b (107) and a chemotactic fragment of C5 (106) enhance the expression of tissue thromboplastin activity by human mononuclear cells. Interaction between the coagulation and fibrinolytic systems in cultures of mononuclear phagocytes has not been addressed. Further characterization of the biosynthesis, intracellular processing, and secretion of tissue thromboplastin will be necessary to determine if inhibitors of this molecule are also expressed by monocytes and macrophages.

Other proteases and their inhibitors are produced by human monocytes and macrophages. Several previous studies suggested that alpha$_1$-antitrypsin, an important serine protease inhibitor, was produced by mononuclear cells (115–118). It is known that alpha$_1$-antitrypsin is synthesized by liver from a 1.4-kb RNA as a 52–55-kd single-chain glycoprotein with 15% carbohydrate attached to three asparagine residues (reviewed in 119). The alpha$_1$-antitrypsin gene is 10.2-kb (120) and localized to chromosome 14 (121–122). It has recently become possible to identify definitely alpha$_1$-antitrypsin biosynthesis by peripheral-blood monocytes, bronchoalveolar and breast macrophages, but not B or T lymphocytes (123). Alpha$_1$-antitrypsin mRNA, similar in apparent size to that in human liver cells, was detected in human monocytes and macrophages by RNA-blot hybridization. Synthesis and secretion of alpha$_1$-antitrypsin was also demonstrated in monocytes and macrophages, but not in lymphocytes. In these biosynthetic labelling experiments, alpha$_1$-antitrypsin was not only identified in its native form but also in forms complexed with serine protease. The serine protease interacting with alpha$_1$-antitrypsin was derived from mononuclear phagocytes. The ratio of alpha$_1$-antitrypsin in native to complexed form, and the apparent size of the alpha$_1$-antitrypsin protease complexes, varied in mononuclear phagocytes from different tissues (blood monocyte, bronchoalveolar, and breast milk macrophages). This suggests independent regulation of serine protease and alpha$_1$-antitrypsin in different local microenvironments.

Several proteases with which alpha$_1$-antitrypsin might interact have been identified in human monocytes. A cell surface serine elastase degrades the amyloid protein, SAA (124), and a secreted elastase mediates plasminogen-independent fibrinolysis (125). It is also possible that neutrophil elastase is taken up from the extracellular fluid by macrophage receptor–mediated endocytosis (126, 127) and later released again into the cell-culture fluid (128, 129). Expression of alpha$_1$-antitrypsin may also be affected by metallo- and thiol-protease, e.g. the murine macrophage elastase (130, 131).

Other factors may affect the balance of alpha$_1$-antitrypsin and protease

in tissues. For instance, another major proteinase inhibitor, alpha$_2$-macroglobulin, is synthesized and secreted by monocytes (132). Alpha$_2$-macroglobulin-protease complexes are internalized by macrophage receptor–mediated endocytosis (133–135). Although alpha$_2$-macroglobulin is similar to alpha$_1$-antitrypsin in inhibition of serine proteases, it also inhibits proteases of the thiol-, carboxyl-, and metalloprotease classes. In addition, the generation of hydrogen peroxide and oxygen radicals by mononuclear phagocytes may influence the alpha$_1$-antitrypsin-protease balance in the local microenvironment. For instance, enhanced expression of oxygen radicals during macrophage activation could oxidize and inactivate alpha$_1$-antitrypsin leading to higher local concentrations of free serine protease.

Expression of both serine protease and alpha$_1$-antitrypsin by mononuclear phagocytes may be similar to the previously mentioned protease-inhibitor systems in providing finely tuned regulation of proteolysis during the initial phases of inflammation and tissue injury. This thesis is based on the observation that lymphokine(s) enhance expression of alpha$_1$-antitrypsin by human monocytes and macrophages (S. Takemura, unpublished), and the pattern of protease-antiprotease expression changes as a function of as yet undefined factors.

Proteases and inhibitors of the coagulation cascade, of a fibrinolytic system, and of the elastase-alpha$_1$-antitrypsin system are only a few examples of proteins that interact with the complement-activating system. Nevertheless, these examples provide ample evidence of the intricate and complex forces that determine the net biological effect of complement and the tissue response to injury/inflammation.

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CONTENTS

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IDIOTYPIC MIMICRY OF BIOLOGICAL RECEPTORS

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INTRODUCTION

This article examines the mimicry of biological receptors by immunoglobulin idiotypes. This mimicry is usually defined empirically by the interaction of antiidiotypic antibodies with epitopes of these receptors. We begin with a definition of the theoretical base and operational terms used in this analysis. For clarity, precise distinctions in terms of specificity and function are made between the different subclasses of antiidiotypic antibodies. Next, we present an overview of the most defined examples of idiotypic mimicry. These systems are to be distinguished from those that regulate lymphocyte function by interaction of antiidiotypic antibodies with lymphocyte antigen receptors. For discussions of these effects we refer the reader to several excellent recent reviews that summarize the roles of antiidiotypic antibodies in the regulation of lymphocyte function, activation, and ontogeny (1–3).

The majority of examples of receptor mimicry are relatively new, and their focus is thus far biased toward the phenomenological and practical use of antiidiotypes as research tools (4, 5). Certainly, the application of antiidiotypic antibodies as specific receptor probes has unique advantages over more conventional ligand-based methodologies. Perhaps most useful is the discovery that antiidiotypes that bind receptors may be generated in the absence of either purified ligand or receptor (6, 7) and that antiidiotypes often mimic the biological activities of ligand upon receptor contact (4, 5, 8, 9). In addition, antiidiotypes by their nature are free of the potentially hazardous effects of ligands and are experimentally maleable. Finally, large

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quantities of purified, monospecific antiidiotopes can now be generated using hybridoma technology.

The major disadvantages of selecting an antiidiotype-based approach to receptor studies are that some idiotype-antiidiotype interactions are genetically restricted (10, 11) and some experimental artifacts arise as a byproduct of cross-reactivity or combinatorial effects of polyclonal anti-idiotype preparations (12). Thus, care must be taken to verify the specificity of antiidiotype binding by using multiple biochemical and immunological methods, and when possible, monoclonal reagents.

The stimulation of idiotypic mimicry of receptors in vivo can perturb the immune network in ways that may affect the maintenance of self-tolerance. Examples of autoimmune diseases believed or suspected to be linked to the presence of antiidiotypic antibodies include myasthenia gravis (13, 14), Grave's disease (15, 16), and insulin-resistant diabetes mellitus (17, 18). A discussion of the potential causative roles and pathogenic consequences of antiidiotypes that display receptor autoreactivity is contained in the closing section of this work.

THE IMMUNE NETWORK

Definition of Terms

The immune system exists as a homeostatic network balanced by interactions between clones of lymphocytes at the level of their specific antigen receptors. The remarkable diversity within and between V_H and V_L gene families is responsible for the vast repertoire of potential antigen receptors and, equally, for the unique idiotypes associated with these receptors. In essence, the network is maintained by the interaction of idiotypes and complementary antiidiotypes. The ground work of this theory was advanced by Jerne (19) in 1974 and with slight modification (20) is widely accepted today. Ample experimental evidence now exists of the presence of antiidiotypes in normal immune responses (21, 22) and of their role in the regulation of antibody synthesis (23) and in the formation of the idiotypic repertoire (1, 24, 25).

Idiotopes are autologous antigenic determinants expressed on immunoglobulin heavy- and light-chain, complementarity-determining regions that are defined serologically by the binding of antiidiotopic antibodies. An idiotype is the set of idiotopic determinants expressed on the V region of a particular antibody. As described in detail in an accompanying chapter of this volume (see Structural Correlates of Idiotopes) most idiotopes, like antigen-binding paratopes, require the participation of both V_H and V_L chains, although some reactivity with isolated chains has been reported (26, 27).

Subclasses of Antiidiotypic Antibodies

The tremendous diversity of the immune system led Jerne (19) to postulate that any external antigen is potentially represented within the immune system as an idiotope determinant. Such idiotopes are called *internal images* of antigens. Nisonoff & Lamoyi (28) have proposed that the term *related epitope* be employed to describe this mimicry in light of the possibility that the paratope of the internal image antiidiotope may represent varying degrees of homology or resemble only a portion of the antigenic epitope.

Theoretically, idiotopes may be located at any antigenically active structure on the immunoglobulin surface created by the complementarity-determining regions. A schematic representation of the different classes of antiidiotopes is presented in Figure 1. Antibodies with internal image activity will be generated only when either (*a*) idiotopes on immunoglobulins of unrelated specificity bear related epitopes (we call this class $Ab2_\beta$ using the convention of Jerne); or (*b*) the paratope of an antibody (Ab1) serves as an idiotope (we call this class $Ab2_\rho$—paratope associated). One should keep in mind that internal image antiidiotopes comprise a minor subset of potential antiidiotypes. Urbain and coworkers have estimated that only 15% of anti–tobacco mosaic virus (anti-TMV) antiidiotypes are internal images (29). The majority of antiidiotypes recognize idiotopes that are either nonbinding-site related (30), or binding-site related, i.e. epitope inhibitable. In keeping with previous nomenclature (32), we call nonsite related antiidiotopes, $Ab2_\alpha$, and site-related antiidiotopes, $Ab2_\gamma$.

Such guidelines make possible prediction of some of the properties of

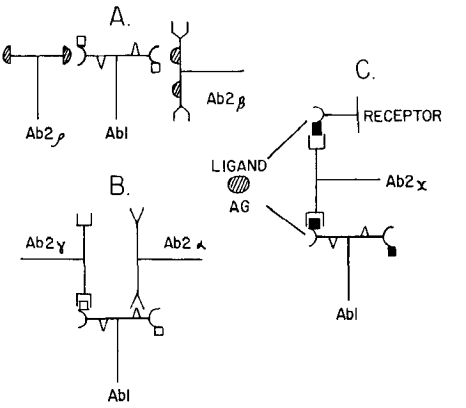

Figure 1 Specificities of antiidiotope antibodies: (A) Internal image–antiidiotopes $Ab2_\beta$ and $Ab2_\rho$; (B) Structural antiidiotopes—$Ab2_\gamma$, binding site related and $Ab2_\alpha$, nonbinding site related; (C) Cross-reactive antiidiotopes–$Ab2_\chi$.

internal image antiidiotypic antibodies. These qualities have been discussed in detail by Nisonoff & Lamoyi (28) and are briefly summarized here with current revisions. The tightness of fit to idealized internal image behavior will depend to a large degree on the extent of epitope relatedness. These properties are: (*a*) Internal image antiidiotopes that contain related epitopes should bind to a substantial proportion of the total anti-X (epitope) population; (*b*) they should recognize anti-X antibodies derived from various strains and species; (*c*) they should compete with antigen for binding to the anti-X binding site; (*d*) only a small fraction of all antiidiotopes should express related epitopes; (*e*) inoculation of an antiidiotope containing a related epitope to that on antigen X should elicit an anti-antiidiotope response (Ab3) that resembles anti-X (Ab1′) and is not species restricted, i.e. antiidiotopes should display antigenic mimicry; and (*f*) in instances where epitope X is involved in the delivery of ligand (antigen) function, e.g. hormone receptor binding, an antiidiotope may either mimic or compete with this function.

Excellent immunological, biochemical, and functional evidence now points to the existence of internal image antiidiotopes. Examples of idiotypic mimicry of biological function include hormone, neurotransmitter, and viral systems; these are discussed in detail in the succeeding sections. Antigenic mimicry by antiidiotypes has also been detected in several diverse systems. Urbain et al (29, 33) were the first unambiguously to describe this phenomenon using polyclonal rabbit antiidiotypic antibodies directed against anti-TMV. Antiidiotypes reacted with essentially all anti-TMV antibodies obtained from rabbits, mice, goats, or chickens, yet they did not bind to anti-DNP, micrococcus, or hemocyanin antibodies. Also, when injected into mice, antiidiotype elicited the synthesis of anti-TMV antibodies. Thus, antiidiotypes to anti-TMV behaved in all respects as if they possessed one or more antigenic determinants of TMV, i.e. contained an epitope(s) related to an epitope(s) on TMV. Results of a similar nature have recently been reported by researchers using monoclonal antiidiotope antibodies to poliovirus type II (34), the mammalian reovirus type 3 (35, 36), sendai virus (37), and phosphorylcholine (38).

Finally, we must consider one additional potential source of receptor-related idiotypic mimicry—that which results when antiidiotypic antibodies recognize cross-reactive structures shared by Ab1 and the physiological receptor of ligand. In this instance the related epitope may be distinct from both the Ab1 paratope and the ligand-binding determinant on receptor. The presence of such cross-reactive structures might be expected of peptide conformations that are closely linked structurally or genetically to sites of equivalent binding specificity on independent receptors, i.e.

antibody as compared to ligand receptors. Similarly, cross-reactivity might be expected if antibody and ligand receptor genes share an evolutionary linkage. These antiidiotopes most probably represent a special subclass of $Ab2_\gamma$ antibodies. This form of antiidiotypic mimicry is presented schematically in Figure 1C. We term these cross-reactive antiidiotopes $Ab2_\chi$.

Because of the predicted linkage of $Ab2_\chi$ idiotopes to the paratope of Ab1 and concordantly of the related epitope on receptor to the ligand binding site, the following general characteristics of $Ab2_\chi$ as compared to $Ab2_\beta$ and $Ab2_\rho$ are expected: (*a*) Because they are binding-site related, antiidiotopes $Ab2_\chi$ should inhibit antigen binding to either Ab1 or receptor; (*b*) they should represent a small fraction of antiidiotypic antibodies; (*c*) the ability of $Ab2_\chi$ to bind anti-X and to induce anti-X (Ab1′) immunity may be species and perhaps strain restricted; and (*d*) binding to receptor would not be expected to mimic ligand function; rather, it would be more likely noncompetitively to inhibit signal delivery by ligand. Although there is not direct proof of the existence of cross-reactive antiidiotopes, the results of several studies may be interpreted in support of their presence. For example, the observation of so-called epibodies, seen in the population of antiidiotypes to human rheumatoid factors (39, 40), represents an analogous cross-reactivity between idiotopes and related epitopes on complementary antigen.

IDIOTYPIC MIMICRY OF RECEPTOR FUNCTION

The preceding paragraphs have outlined the structural and biological characteristics of internal image and cross-reactive antiidiotypic antibodies in general terms. We now address specific examples that demonstrate functional idiotypic mimicry of biological receptors or, correspondingly, antiidiotypic mimicry of ligand. It is important to reemphasize that the existence of internal image as contrasted to cross-reactive antiidiotypy is difficult to prove and should be attempted by using a combination of those characteristics of antiidiotypes listed previously and, when possible, monoclonal reagents. When possible, we distinguish these classes of antiidiotypic mimicry in the examples that follow.

Hormone and Vitamin Receptors

RETINOL-BINDING PROTEIN RECEPTORS Sege & Peterson (41) were the first to demonstrate idiotypic mimicry experimentally at the level of biological receptors. Working directly from assumptions based on Jerne's network hypothesis, these workers showed that a crude antiidiotypic antibody preparation generated against antiretinol-binding protein (RBP) im-

munoglobulin did bind specifically to human prealbumin, a natural carrier of RBP. Idiotype (Ab1) was prepared by injection of rats with RBP, and anti-RBP antibodies were purified from sera by affinity chromatography on RBP coupled to sepharose beads. Affinity-purified anti-RBP was used to immunize rabbits, and antiidiotypic antibodies were isolated by chromatography over protein A sepharose, then extensively absorbed with rat immunoglobulin and RBP-sepharose to remove xenogeneic cross-reactive antibodies and free RBP. These antiidiotypes bound specifically to labeled human prealbumin or anti-RBP antibodies, and this binding was inhibited by the addition of either unlabeled anti-RBP antibody or RBP itself, which suggests that the antiidiotype and RBP bound to identical or overlapping sites on prealbumin.

Confirmation of the specificity of anti-RBP antiidiotypes was achieved by the demonstration of antiidiotype binding to RBP receptors on the surface of rat intestinal epithelial cells. This binding was blocked by the addition of anti-RBP; however, inhibition by RBP was not reported. The binding of antiidiotype was also shown to inhibit RBP-mediated uptake of ^{3}H-retinol by intestinal epithelial cells (8), reinforcing the notion that antiidiotype bound to the RBP-recognition site. It is not possible at this time unambiguously to determine the class of these antiidiotypes; however, their cross-species reactivity and inhibitory effects favor classification as $Ab2_{\beta}$.

INSULIN RECEPTORS Sege & Peterson were also the first to demonstrate antiidiotypic mimicry of physiological signals delivered by ligand (8). Xenogeneic antiidiotypic antibodies to antibovine insulin were prepared as described above for RBP. These antiidiotypes bound specifically to antiinsulin antibodies, and this binding was inhibited by insulin but not RBP. Antiinsulin antiidiotypes, but not preimmune immunoglobulins, inhibited the binding of ^{125}I-insulin to epididymal fat cells in a dose-dependent fashion. This inhibition was reversed by the addition of unlabeled antiinsulin antibody. Most significantly, antiidiotype binding to rat thymocyte cells stimulated the uptake of α-aminoisobutyric acid in a way reminiscent of the effects of insulin. The amount of antiidiotype needed to induce equivalent levels of uptake was, however, 1000-fold (by weight) beyond that of insulin, and control immunoglobulin also stimulated a low but reproducible increase in uptake (8, 12). These results reflect typical problems associated with the demonstration of idiotypic mimicry. While discrepancies in specific bioactivity could be explained by the likelihood that internal image antiidiotypes comprise only a small fraction of the total antiidiotypic population, confirmation of functional mimicry awaits the precise measurement of antiidiotypic effects in an insulin-dependent signal

transmission assay. We suggest that the stimulation of insulin receptor phosphorylation in vitro or in vivo would provide a definitive assay of mimicry.

More recently, Shechter and coworkers (42, 43) have shown that mice immunized with bovine or porcine insulin spontaneously develop antibodies to both insulin and the insulin receptor. Antiinsulin receptor antibodies were identified as antiidiotypes because they specifically bound to guinea-pig-insulin antibodies (42) and because their insulin-like effects, listed below, were inhibited by affinity-purified mouse-antiinsulin idiotypes (44). The possibility that these effects were due to insulin contamination was excluded by repeated passage of antibodies over insulin-immunoadsorbent columns. Antiidiotypic antibodies blocked the binding of ^{125}I-insulin to fat cells, stimulated glucose oxidation and lipogenesis, and inhibited lipolysis, all of which are insulin-induced effects. These observations strongly suggest the presence of internal image Ab2ρ or perhaps Ab2β antibodies to insulin receptors. Confirmation of this requires immunological demonstration of the ability of antiidiotype to induce antiinsulin antibodies across species barriers. These studies also provide substantial insight into the potential role(s) of spontaneously generated antiidiotypes in autoimmunity. The relevance of this observation is discussed more fully in a succeeding section.

THYROTROPIN RECEPTORS The application of antiidiotypic antibodies as probes of thyroid stimulating hormone (TSH) receptors has been conducted by Farid and coworkers (45). These studies were begun in an attempt to elucidate both the structure and the function of the TSH receptor, and the role of anti-TSH receptor antiidiotypes in the etiology of autoimmune Grave's disease.

Anti-TSH antibodies were isolated from rats immunized with either human or bovine TSH (45). The anti-TSH immunoglobulin fraction was purified by elution from a TSH-affinity column. Rabbits were immunized with anti-TSH and the resultant immunoglobulins were purified and absorbed with preimmune rat immunoglobulin. The presence of antiidiotypes to anti-TSH in this polyclonal population was demonstrated by their ability to inhibit the binding of ^{125}I-TSH to anti-TSH antibodies and to thyroid plasma membranes. Antiidiotypic antibodies themselves displayed specific, saturable, and high-affinity binding to purified thyroid plasma membranes (5). Antiidiotype was also shown to mimic many of the biological effects of TSH on thyroid cells including: (*a*) stimulation of membrane-associated adenylate cyclase activity in the presence of guanyl nucleotides; (*b*) inhibition of ^{131}I uptake by thyrocytes when briefly pulsed (10 min) with antiidiotype; (*c*) stimulation of ^{131}I thyrocyte uptake upon

prolonged incubation (4 hr); and (*d*) organization of cultured thyrocytes from disperse, flattened monolayers into follicles containing rounded cell aggregates (45, 46). Results of a similar nature have been reported by Beall et al (47), and recently Baker et al (48) have prepared antiidiotypes to anti-TSH hormone receptors that inhibit TSH-mediated adenylate cyclase stimulation.

Anti-TSH antiidiotypes have also been used to characterize the TSH receptor biochemically. Radiolabeled antiidiotype bound a M_r 197,000 band on western blots of thyroid plasma membranes run on SDS-polyacrylamide gels under nonreduced conditions (5, 48). This is in good agreement with a previously reported holoreceptor of M_r 200,000 obtained by ligand isolation (49). This same band was identified by labeled TSH, but not human chorionic gonadotropin or insulin, and preincubation of blotted paper with unlabeled TSH blocked the binding of a labeled antiidiotype to M_r 197,000 (5, 48). Antiidiotype did not bind to receptor subunits when gels were run under reduced conditions; this suggests that idiotypic mimicry reflected binding to epitopes present on the native conformation of TSH.

Based on these findings, monoclonal anti-TSH antibodies that recognize either the α or β subunits of human TSH were used to induce antiidiotypes (48, 50). The characteristics of these antiidiotypes were similar to those previously described with the exception that anti-αTSH and anti-βTSH antiidiotypes displayed additive or synergistic effects of mimicry. For example, binding to the holoreceptor on western gel blots and stimulation of adenylate cyclase activity were only detected when anti-α and anti-β antiidiotypic antibodies were added simultaneously and in equal proportions (5, 51). In addition, stimulation of ^{131}I uptake in thyroid epithelial cells by anti-βTSH antiidiotype was substantially enhanced by the addition of anti-αTSH antiidiotype, which had no effect on its own. These observations have enabled a partial dissection of the mechanism of TSH signal delivery. Farid and co-workers have proposed that the action of TSH is most probably linked to the effects of α and β subunits cooperating to deliver a single stimulatory trigger, as opposed to two independent stimulatory events. According to this model, low-affinity binding of the noncatalytic αTSH subunit induces a conformational change in the receptor that enhances the binding affinity of the catalytic βTSH subunit, which in turn stabilizes αTSH subunit binding.

Neurotransmitter Receptors

β-ADRENERGIC RECEPTORS The use of natural ligand analogues in the preparation of an antiidiotypic antibody membrane receptor probe has been elegantly demonstrated in studies of β-adrenergic receptors (9, 52–56).

Antibodies prepared to the β-adrenergic antagonist alprenolol were used as immunogens to generate antiidiotypes that behaved in one instance as an agonist (9, 52) and in another as an antagonist (55).

Homcy et al (55) have isolated antiidiotype to affinity-purified anti-alprenolol antibody. These antiidiotypes inhibited the binding of ^{3}H-alprenolol to its idiotope and possessed antagonist activity. Antiidiotypic antibodies behaved as competitive inhibitors of β-agonists as measured by direct binding assay and by their effects on basal- and agonist-stimulated adenylate cyclase.

Using a similar protocol, Strosberg and co-workers have prepared polyclonal, and more recently monoclonal (mAb2B4), antiidiotypic anti-alprenolol antibodies that bind to β-adrenergic receptors and inhibit ^{3}H-alprenolol binding. Polyclonal and mAb2B4 antiidiotypes had potent agonist activity (9, 52). Antiidiotypic antibodies did bind to turkey erythrocytes, which possess β-adrenergic receptors, but not to human or sheep erythrocytes, which are devoid of β-adrenergic receptors (52, 54). This binding was inhibited by preincubation of antiidiotypes with antialprenolol antibodies or by pretreatment of cells with β-adrenergic agonists or antagonists (9, 52). These workers also utilized antiidiotypic antibodies to characterize receptors on A431 human epidermoid cells biochemically (9). mAb2B4 bound to a single M_r 55,000 protein on western blots of isolated membranes, in good agreement with the reported M_r 50,000–67,000 of β-receptors (57, 58). Further evidence that mAb2B4 recognized β-receptors was achieved by the demonstration that immunoprecipitation of receptors from detergent solubilized A431 cells removed 80% of the alprenolol-binding activity from the supernatant, while immunoprecipitations performed with control mAb had no effect (9).

Studies have not been conducted to determine whether idiotypic mimicry of β-receptors is the product of internal image or cross-reactive antiidiotypes; however, a series of valuable immunologic experiments illustrated the reciprocal cycling of idiotype and antiidiotypic antibodies. When antialprenolol antibodies were injected into rabbits, antiidiotypes appeared in brief 2–3-week recurrent peaks (53). These observations resemble those reported in several other systems (3, 59). The disappearance of antiidiotype was correlated with the appearance of autologous anti-antiidiotypes that possessed catecholamine-ligand-binding activity. Initially anti-antiidiotype (Ab1′), purified by antiidiotype affinity chromatography, bound labeled alprenolol with a higher affinity than did idiotype. Interestingly, in succeeding weeks the affinity of anti-antiidiotypes decreased substantially relative to the initial antialprenolol antibodies (idiotype) even when the animals' immunity was intermittently boosted with antiidiotype. These observations are in direct contrast to well-

established observations of the selective increase of antibody affinity to ligand during the ontogeny of immune responses (60).

These results suggest that the immune network may selectively modulate the affinity of autoreactive clones in a unique fashion to limit the extent of autoreactive expansion. Studies that directly compare the binding affinities of "normal" versus autoreactive antiidiotypes and anti-antiidiotypes will be necessary to resolve this issue. The work of Strosberg et al also points out that continuous monitoring of antiidiotype activity is a major practical concern for the successful isolation of antiidiotypic antibodies because the timing of antiidiotype cycling varies greatly among individual animals.

ACETYLCHOLINE RECEPTORS Investigation of the possible interaction of antiidiotypes and acetylcholine receptors (AChR) began as an extension of work conducted to elucidate the causes of myasthenia gravis (MG). Patrick & Lindstrom (61) first demonstrated that immunization with purified AChR led to clinical symptoms of MG. These and other studies (62, 63) formed the basis for an animal model of MG, known as experimental myasthenia gravis (EMG), and established a firm linkage between the occurrence of MG and the presence of AChR antibodies (64, 65).

In view of these findings and observations of idiotypic mimicry discussed previously, Erlanger and coworkers were prompted to investigate the potential pathological effects of anti-AChR antiidiotypic antibodies. Polyclonal antiidiotypes were produced by immunizing rabbits with affinity-purified rabbit antibodies to BisQ, a synthetic AChR agonist. Sera from these animals bound AChR on rat or eel cells, and binding was inhibited by BisQ (14). These anti-AChR antibodies were not, however, directly demonstrated to be antiidiotypes to anti-BisQ. Interestingly, two of three rabbits immunized showed signs of muscle weakness typical of EMG, although this was to a milder extent than that seen following immunization with AChR. The severity of EMG symptoms in the two rabbits correlated well with their anti-AChR titers. The rabbit that had a significant anti-AChR titer showed no signs of muscle weakness; this indicated that multiple factors affect the ultimate sensitivity to anti-AChR antibody.

As a sequel to these observations Cleveland et al (66) have isolated and characterized mAb anti-AChR antiidiotopes from mice immunized with BisQ–bovine serum albumin conjugates. One mAb antiidiotope, termed F8-D5, was shown specifically to bind rat and eel AChR, and this binding was inhibited by the presence of BisQ. Whether the administration of mAb F8-D5 induces EMG was not reported.

Cellular Receptors for Virus

THE MAMMALIAN REOVIRUS RECEPTOR Antiidiotypic antibodies have been used as probes of the cell biology and immunology of the mammalian

reovirus in our laboratories. These studies employed the unique properties of antiidiotypes to study: (*a*) The cellular distribution and biochemistry of reovirus receptors; (*b*) the parameters of reovirus receptor binding and internalization; (*c*) the structural and functional relationships of virus receptors on neurons and lymphocytes to antigen receptors for virus, i.e. idiotypes on B and T lymphocytes; and (*d*) the use of mAb antiidiotope antibodies as viral vaccines.

The binding of reovirus to its cellular receptor is governed solely by the specificity of the minor outer capside protein S1, which also mediates virally induced hemagglutination (67–70). The S1 protein is thus often referred to as the viral hemagglutinin (HA). In addition to tissue tropism, the reovirus HA determines the serotype specificity of antibodies, cytolytic T cells, and in vivo delayed-type hypersensitivity reactions (67, 71–73).

Preparation of antiidiotypic antibodies The production, purification, and screening of polyclonal reovirus type-3(HA3)–specific antiidiotypic antibodies have been described by Nepom et al (6). Briefly, immunoglobulins from mice immunized with type-3 virus were first absorbed with type-1 virus and the recombinant virus 3HA1 (a type-3 virus with a type-1 HA) to remove all antibodies not directed against HA3. Immunoglobulins were then absorbed with preimmune mouse immunoglobulin to remove xenogeneic reactive antibodies. Rabbits were immunized with these polyclonal anti-HA3-specific antibodies, and the presence of anti-HA3 antiidiotypes in the resultant sera was verified by radioimmunoassay. A panel of monoclonal anti-HA3-specific antibodies was then screened for the ability to inhibit the binding of type-3 antiidiotype to anti-HA3 antibodies. One neutralizing mAb, termed 9BG5, inhibited 83% of antiidiotype binding, indicating that this epitope is immunodominant in the type-3–antiidiotype response.

Based on this finding, 9BG5 immunoglobulin was used as an immunoabsorbent to affinity purify HA3-specific antiidiotypes from crude immunoglobulin fractions. Indirect immunofluorescence staining with purified antiidiotype demonstrated that the pattern of antiidiotype binding mimicked the tissue tropism of reovirus type 3, but not of type 1, on both central nervous system and lymphoid targets (6, 74). When lymphocytes were separated into reovirus-binding and -nonbinding populations, purified antiidiotype binding was restricted to only those cells that bound reovirus. Results of a similar nature were obtained by double fluorescence labeling of reovirus type 3 and 9BG5 purified antiidiotype (74). We have also shown that antiidiotype can mimic viral functions upon binding to target cells. For example, antiidiotype induced the activation of suppressor T cells in a manner indistinguishable from that seen with intact virus (74). Taken together these results indicate that the epitope on the type-3

Table 1 Antiidiotype inhibition of reovirus binding is restricted to idiotype positive lines[a]

	Cell lines			
	Idiotype positive		Idiotype negative	
	R1.1	R1.E	EL.4	P815
Untreated	3319 ± 530	3355 ± 348	7355 ± 1832	15,380 ± 549
Treated with antiidiotype	317 ± 416	365 ± 183	6022 ± 77	13,356 ± 662
% Inhibition of viral binding	90.4	89.1	18.1	13.2

[a] Binding studies were conducted at 4°C by the addition of 25,000 cpm ^{35}S-labeled reovirus (2×10^6 cells) for 1 hr. The effect of antiidiotype was determined by preincubation of cells with purified antiidiotype for 30 min prior to virus addition. Untreated controls were without antibody addition. Binding is expressed as cpm bound per 2×10^6 cells ± SEM of triplicate samples. From Gaulton et al (4).

hemagglutinin recognized by mAb 9BG5 is involved in the binding of type-3 virus to its cellular receptor.

More recently, a syngeneic murine monoclonal type-3-specific anti-idiotope has been isolated following immunization of Balb/c mice with the 9BG5 mAb (7, 67, 75). Anti-HA3-specific mAb antiidiotopes were initially screened for the ability to inhibit the binding of 9BG5 to purified ^{125}I-HA3 protein in a competitive radioimmunoassay (7). One mAb, termed 87.92.6, that blocked binding was selected for further study. The binding of 87.92.6 to a panel of cells mirrored both the type-3 and polyclonal antiidiotype patterns.

Direct evidence that mAb antiidiotope recognized the reovirus type-3 receptor was achieved by the demonstration that prior incubation with 87.92.6 specifically inhibited the binding of type-3 virus to R1.1 thymona cells by 70–90% (Table 1; see also 75, 76). In addition, recent evidence indicates that mAb antiidiotope inhibits the infectivity of reovirus type 3 on neuronal targets (M. A. Dichter, G. Mitchell, H. Weiner, B. Fields, J. Noseworthy, G. Gaulton, M. Greene, manuscript submitted). Infectivity studies were conducted using three-week-old rat-neuronal-cell cultures. Only neurons specifically bound 87.92.6, and greater than 90% of all neurons bound antiidiotope. When mAb antiidiotope (200 μg) was added to cultures for 60 min prior to the addition of virus (5×10^4 plaque-forming units), a 75% reduction in the number of viral replicating bodies was seen relative to control antibody. If antiidiotope was mixed with virus and then added to neuronal cultures, a 40% reduction in viral replication was seen. As mAb 87.92.6 does not bind reovirus (G. Gaulton, unpublished information), the blocking effects of mAb antiidiotope are a result of specific competition for binding to the cellular virus receptor.

Isolation of reovirus receptors The isolation and characterization of reovirus receptors was accomplished using both polyclonal and mono-

clonal antiidiotypes. Reovirus receptors were isolated from surface-radioiodinated-R1.1 thymoma cells by precipitation of detergent-soluble cell extracts with either purified polyclonal antiidiotype, mAb 87.92.6, or type-3 reovirus particles. An autoradiograph of immunoprecipitates run on SDS-polyacrylamide gels is presented in Figure 2. Polyclonal antiidiotype (lane 1) routinely precipitated a single M_r 67,000 protein, not seen in controls, which is also the major band of monoclonal (lane 2) and virus (lane 3) precipitations (76, 77). The additional bands seen in monoclonal and virus precipitates result from a combination of less efficient precipitation conditions and partial degradation of receptors during isolation.

Immunoprecipitations have also been performed on a variety of different cell types and donor species including several neuronal lines. In all instances receptors were found to be of identical molecular weight (77). More detailed analyses indicated that purified receptors were sensitive to digestion by protease and neuraminidase, shown in lane 4 of Figure 2, and that the receptor is monomeric with a slight charge heterogeneity of pI 5.8–6.0.

These observations are strengthened by the results of antiidiotypic antibody hybridization to western blots of purified R1.1 cell membranes run on SDS-polyacrylamide gels. ^{125}I-labeled reovirus type 3 and polyclonal and monoclonal antiidiotype all bound to a similar band of M_r 67,000 that was not seen in hybridizations conducted with either normal rabbit immunoglobulins, antibovine serum albumin, or control mAb (76, 77).

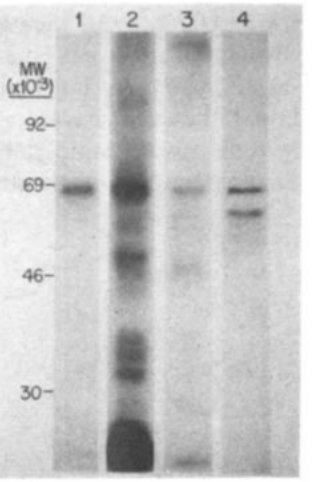

Figure 2 Electrophoretic analysis of antiidiotype and virus precipitates. ^{125}I-labeled R1.1 detergent lysates were precipitated with either affinity-purified rabbit antiidiotype (lane 1), mAb 87.92.6 antiidiotope (lane 2), or type 3 virus (lane 3). The product of neuraminidase treated polyclonal antiidiotype immunoprecipitates is shown in lane 4. From Co et al (77).

Purified mAb 87.92.6 has recently been used to quantitate the number of receptors on target cells (76, 77). These analyses demonstrate that equivalent numbers of receptors are detected by antiidiotope and virus—approximately 50,000–75,000 sites per cell, which approaches saturated packing of virus on the surface (69, 78).

Structural similarities of reovirus and β-adrenergic receptors Data from a number of systems indicate that many viruses utilize integral membrane proteins, which provide essential cellular functions, as specific viral-binding proteins. For example, the membrane receptor for lactate dehydrogenase virus on macrophages has been identified as the Ia molecule (79), the Epstein-Barr virus has been shown to bind to the C3d receptor CR2 on B lymphocytes (80, 81), and the T-cell marker OKT4 serves as an attachment site for HTLV-3 (82, 83). Using anti-HA3 antiidiotypic antibodies, experiments from our laboratory indicate that the β-adrenergic receptor serves as a reovirus-type-3 attachment site.

We initially observed that both the reported molecular weight and the tissue and distribution of the mammalian β-adrenergic receptor matched those of the mammalian reovirus type-3 receptor (57, 70, 71, 84, 85). Based on this, more detailed studies were conducted (86) and are summarized here. Antiidiotypic antibodies specifically immunoprecipitated conventionally purified (by β-ligand affinity chromatography) β2-adrenergic receptors. As shown in Figure 3, the two-dimensional gel patterns of purified reovirus (panel A) and β-adrenergic (panel B) receptors are indistinguishable. The partial trypic digestion fragments of purified reovirus and β-adrenergic receptors run on SDS-polyacrylamide gels were also identical, indicating that receptor homology extends beyond the ligand–virus binding domain. To determine whether antiidiotype binds functionally active β-adrenergic receptors, β-ligand-binding studies were conducted using the radiolabeled β-antagonist ^{125}I-iodohydroxybenzylpindolol (IHYP). As shown in Figure 4, we have analyzed both the amount of membrane-bound IHYP that

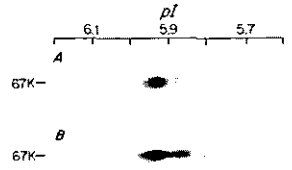

Figure 3 Comparison of purified reovirus type 3 and β-adrenergic receptors by two-dimensional gel electrophoresis. Panel A, reovirus receptor isolated from R1.1 thymoma cells by antiidiotype immunoprecipitation. Panel B, β-adrenergic receptor ligand-affinity purified from calf lung. From Co et al (86).

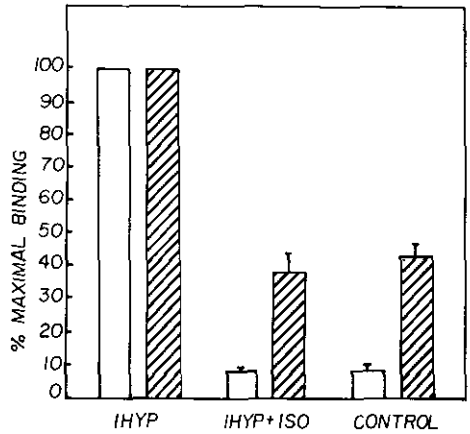

Figure 4 Binding of β-adrenergic ligands to purified and cellular reovirus receptors. The extent of ^{125}I-HYP binding and competition by isoproterenol (ISO) was assessed by measuring antiidiotype (open bars) and by the binding of IHYP to antiidiotype purified receptor (hatched bars). Control refers to immunoprecipitations using preimmune rabbit immunoglobulin. From Co et al (86).

coprecipitates with reovirus receptors and the binding of IHYP to antiidiotype-purified reovirus receptors. In both instances, the specificity of IHYP binding was verified by inhibition in the presence of the unlabeled β-agonist isoproterenol to control levels, which ranged from 60–90% inhibition for receptor and whole cells assays, respectively. Competitive binding analyses have indicated that IHYP displays saturable binding to purified receptors and that, as expected from coprecipitation data, reovirus and antiidiotype bind to a separate site on receptors from that recognized by β-adrenergic ligands (G. Gaulton, manuscript in preparation).

No studies have been conducted that offer a clear picture of whether antireceptor antibodies recognize an epitope found on both β1- and β2-adrenergic receptors. Although the studies described above were conducted using β2 receptors isolated from calf lung, reovirus preferentially infects heart and muscle tissues, which express predominantly β1 receptors (58, 84, 85). In addition the number of cellular reovirus and antiidiotypic antibody-binding sites is 5–10-fold greater than typical β2-receptor levels. This suggests either the presence of previously undetected low affinity β-sites or that antiidiotypes recognize an epitope present on a larger family of β, and perhaps other, catecholamine receptors.

Modulation of the immune network by syngeneic monoclonal antiidiotope antibody As we indicated previously, the network theory predicts that immunization with antiidiotypic antibodies (Ab2) will elicit an anti-

antiidiotypic (Ab3) response. In the case of internal image antiidiotope antibodies (Ab2 β and ρ), a subset of Ab3 will resemble the initial antibody (Ab1) in the specificity of its epitope binding (Ab1'). Regulation of antibody responses in the network has been shown to include coordinate regulation of idiotype- and antiidiotype-bearing T cells (1, 2, 87, 88). Thus, if mAb 87.92.6 represents an internal image of reovirus type-3 hemagglutinin, immunization with 87.92.6 should induce HA3 specific antireovirus immunity at both the T- and B-cell levels.

These predictions have been verified by our laboratories. Immunization of syngeneic mice with purified mAb 87.92.6 stimulated potent, dose-dependent and type-3-specific anti-viral responses, as determined by cytolytic-T-cell, delayed-type-hypersensitivity, helper-T-cell, and antibody assays (35, 36, 89). The exquisite specificity of these responses is typified by the results presented in Table 2 for elicitation of a delayed-type hypersensitivity response.

Immunization with mAb 87.92.6 antiidiotope primed the host for the expression of a hypersensitivity response only upon challenge with either type-3 reovirus (HA3) or the recombinant virus 1HA3 (type-3HA on a type-1 background), but not with either type-1 reovirus (HA1), the recombinant 1HA3 (type-1HA on a type-3 background), or the type-3 variant virus K, which differs only in the loss of the epitope recognized by 9BG5 (idiotope).

The use of mAb 87.92.6 as an antiviral vaccine has been extended to include immunizations of a variety of mouse strains and across species barriers. In each instance 87.92.6, when administered with adjuvant,

Table 2 Specificity of syngeneic delayed-type hypersensitivity responses elicited by mAb 87.92.6 antiidiotope[a]

Immunization	Challenge	DTH response
Type 3 virus	Type 3	37.8±2.3
Antiidiotope	Type 3	25.5±1.7
Antiidiotope	Type 1	8.5±1.5*
Antiidiotope	1 HA3	27.3±2.0
Antiidiotope	2 HA1	9.5±1.6*
Antiidiotope	Variant K	11.8±1.7*
Saline	Type 3	8.5±1.5*

[a] DTH responses are expressed as the mean footpad swelling ± SEM 24 hours after challenge, and day 5 after immunization, with virus as indicated (units of mm $\times 10^{-2}$).

* $p < 0.005$.

stimulated a potent and type-3-specific neutralizing antibody response (G. Gaulton, manuscript in preparation). Preliminary results also indicate that these animals are resistant to viral-induced pathogenesis and mortality.

Studies conducted using the reovirus-type-3 antiidiotype system demonstrate the experimental versatility and corresponding practical importance of internal image antiidiotype antibodies. The demonstration that virally induced antiidiotypic antibodies can function as anticatecholamine receptor autoantibodies has provocative implications for the etiology of several autoimmune diseases that are indirectly linked to the presence of virus (90–93).

RETROVIRUS RECEPTORS

Using an analysis similar to that described for reovirus receptors, Ardman et al (94) have prepared antiidiotypic antibodies that react with the cellular receptor for a family of polytropic leukemogenic retroviruses. The binding of leukemogenic retrovirus to target cells occurs though the viral envelope glycoprotein gp70 (95–97), which is unique for each cloned viral recombinant. A monoclonal antibody, 1416, was prepared that reacted specifically with a cloned polytropic retrovirus termed P1, but not with two other recombinant viruses (P2 and P5) or with endogenous ecotropic and xenotropic viruses.

Antiidiotypic antibody to 1416 was prepared in rabbits and extensively absorbed with mouse immunoglobulin, then affinity purified over a mAb 1416-sepharose column. Eluted antiidiotype bound selectively to 1416, and this binding was completely inhibited by P1 virus or P1 gp 70 (in a two-fold excess by weight), but not by P2 virus. Thus, at least a portion of this antiidiotype was binding-site related. Surprisingly, immunofluorescence analysis indicated that antiidiotype bound to both P1- and P2-induced thymic leukemias and also bound to several leukemia cell lines of both T- and B-cell origin. Antiidiotype did not bind to normal lymphoid cells or to mitogen-stimulated thymocytes. These data indicate that anti-gp70 antiidiotypes recognized either a family of shared idiotypic structures on different anti-gp70s and on gp70 receptors or, as a result of the potential imprecision of related epitopes, recognized a common structural element at the gp70 binding site.

Immunoprecipitations of labeled P1-thymic-leukemia cells identified proteins of M_r 70,000, 63,000, and 55,000. Proteins of M_r 70,000 and 55,000 were also seen in immunoprecipitates from antiidiotype-binding cells, whereas the M_r 63,000 protein was not uniformly observed. Depending on the in vivo binding specificity of anti-gp70 antiidiotypes, these studies may have widespread diagnostic and therapeutic applications.

Receptors on Leukocytes

FACTOR H RECEPTOR Factor H (βIH-globulin) is an important controlling protein in the alternative pathway of complement activation. Factor H binds specifically to receptors on B lymphocyte membranes and triggers blastogenesis and release of factor I (98, 99). Factor H also binds to complement component C3b and acts as a cofactor for factor-I cleavage of bound C3b (100, 101). Antiidiotypic antibodies to antifactor H were induced in rabbits by injection of purified $F(ab')_2$ goat antifactor H; they were then isolated by affinity chromatography over an antifactor-H immunoadsorbent (102). ^{125}I-antiidiotype did bind specifically to cells known to bind factor H (B cells and sheep erythrocytes coupled with C3b); it also blocked the binding of ^{3}H-factor H to these same cells. Antiidiotype mimicked factor-H activity by stimulating the release of factor I from B cells and by potentiating fluid-phase-C3b cleavage in the presence of factor I. These results demonstrated for the first time that C3b and factor-H membrane receptors have similar H-binding sites.

Antiidiotypic antibodies to antifactor H were also used in the isolation of the factor-H receptor from B cells. The immunoprecipitates, which were of detergent-solubilized B-cell membranes labeled with ^{3}H-leucine, contained bands of M_r 100,000 and 150,000 on nondenaturing gels; these converted to a single M_r 50,000 species after reduction. Verification that these proteins comprised the factor-H receptor was achieved by the observation of identical bands from eluates of factor H–agarose affinity columns.

NEUTROPHIL-FORMYL-PEPTIDE RECEPTORS Marasco & Becker (103) used polyclonal antiidiotypic antibodies as probes of the neutrophil receptor for the chemotactic formyl peptide, fmet-leu-phe. Affinity-purified rabbit anti–fmet-leu-phe antibodies were used to elicit xenogenic antiidiotypic antibodies in goats, guinea pigs, and mice. After absorption with rabbit immunoglobulin, goat antiidiotypes were shown to bind anti–fmet-leu-phe, to block the binding of fmet-leu-phe to neutrophils, and to bind rabbit neutrophils. Receptor number and specificity predicted with antiidiotype were comparable to values obtained using labeled ligand. A hierarchy of antiidiotype production by species was seen, with goat antiidiotypes displaying the highest activity. Unfortunately, because preimmune immunoglobulins themselves induced chemotaxis, it was not possible to demonstrate mimicry of biological activity. These observations suggest that antiformyl peptide antiidiotype may contain $Ab2_\chi$ antibodies that display cross-reactivity to idiotypes on antiformyl peptide and epitopes on formyl peptide receptors.

Preliminary Examples of Mimicry

Based on the successes of the work discussed in preceding sections, numerous investigators have begun to utilize the phenomenon of idiotypic mimicry as an approach to the investigation of biological receptors. Accordingly, we include here a brief summary of the most recent and preliminary examples of receptor idiotypic mimicry.

Amit et al (104) have prepared polyclonal antiprolactin antiidiotypic antibodies that inhibit to 85% the binding of ^{125}I-prolactin to antiprolactin (idiotype). At a 10-fold higher concentration, antiidiotype noncompetitively inhibited 30% of prolactin binding to membrane receptors. Antiidiotypes were also shown to mimic prolactin biological activity by stimulating α-lactalbumen synthesis and secretion.

Schulz & Gramsch (105) have prepared antiidiotypic antibodies that recognize opioid receptors by immunization of rabbits with a murine mAb (3-E7) that recognizes the N-terminal site of many naturally occurring opioid peptides. Antiidiotypes were purified from sera by passage over 3-E7 affinity columns. Of 12 different rabbit antiidiotypes tested, 1 was found that inhibited 60–70% of ^{3}H-diprenorphine binding to solubilized rat brain receptors. This same antiidiotype preparation also inhibited PGE_1-driven cyclic AMP synthesis by neuroglioma cell hybrids in vitro. This data suggests that anti–3-E7 antiidiotypes have an agonist activity, since the inhibition of cyclic AMP synthesis resembles that seen with opioid agonists.

Several groups have recently reported the isolation of components of membrane transport systems using antiidiotypic antibodies. These include the mammalian glucose transporter on rat myoblasts (5) and red blood cells (106), and bacterial dicarboxylic acid transport components (5). Mimicry or inhibition of biological activity has not been reported in any of these systems.

AUTOIMMUNITY AS A CONSEQUENCE OF IDIOTYPIC MIMICRY

Our knowledge of the immune system, especially through observations of the immune network, and class-I and -II MHC-restricted lymphocyte interactions, illustrates that autoreactivity is a normal and essential component of immune regulation. It is thus tempting to speculate that autoimmunity is in part a consequence of abnormal immune regulation. Data relevant to this proposal are critically reviewed in several recent articles (107–110). We restrict our discussion here to evidence that indicates that antiidiotypic antibodies can act as effectors of autoimmune disease. We

also present a hypothetical model for the induction of autoimmunity that encompasses both clinical observations and experimental demonstrations of idiotypic mimicry.

Occurrence of Receptor-Binding Antiidiotypes in Experimental Autoimmunity

In the previous section we presented several examples that demonstrate that one potential consequence of idiotypic mimicry of epitopes lying at or near the ligand binding site of biological receptors is the production of antiidiotypic antibodies that bind these receptors. The association between the induction of antiidiotypic, antireceptor antibodies and autoimmune disease is a logical one in view of the examples of human disease closely related to the presence of antireceptor antibodies. These include Grave's disease, in which TSH receptors are hyperactivated by antireceptor antibodies (11), myasthenia gravis, in which acetylcholine receptors are blocked by antibody (108), and certain forms of insulin-resistant diabetes mellitus associated with abnormal regulation of insulin receptors by antibodies to these receptors (109).

One might well ask at this point whether there are any specific examples of ligand-induced auto-antiidiotypic antibodies that can produce autoimmune disease. The answer is a qualified yes. We have already discussed the work of Erlanger et al (14, 66) which demonstrated that: (*a*) antiidiotypic anti-AChR antibodies arise spontaneously following immunization with an AChR agonist (BiSQ); and (*b*) the induction of antiidiotypic anti-AChR antibodies in a xenogeneic system often stimulates MG-like pathology.

Similar results have been reported by Shechter et al (42–44) for antiinsulin antiidiotypes and diabetes-like disease. These workers demonstrated the spontaneous autochthonous production of antiinsulin receptor antiidiotypes in mice following immunization with beef or pig insulin (42). When individual mice were followed for diabetes-like symptoms such as intolerance to glucose loading and hyperglycemia, a very tight correlation was seen between disease incidence and antiidiotypic antireceptor titers (43, 44). However, since these mice contained antiinsulin-receptor antibodies that were not antiidiotypic, an absolute correlation was not possible.

The observations of idiotypic mimicry of receptors for virus also have implications for autoimmune pathology. A number of viruses have been associated with an increased incidence of specific autoimmune disease. The best examples of this include β-islet tropic viruses such as coxsackie B-4, encephalomyocarditis, and reovirus, and the occurrence of insulin-dependent diabetes mellitus (90–93, 109). In these instances antiidiotypic, antiviral receptor antibodies would be expected to constitute either

the primary active pathological agent or, more likely, to stimulate a widespread antireceptor or antitarget-cell immune response following limited antiidiotype-linked cell disruption.

Antiidiotypic Antibodies in Human Autoimmune Sera

If the predictions advanced above are true, then we might expect to see significant antiidiotype, and particularly antireceptor antiidiotype levels in the sera of patients with antireceptor-linked autoimmune diseases such as MG, Grave's disease, or insulin-dependent diabetes mellitus. The most thorough investigation of antireceptor antibodies and antiidiotypes in autoimmunity has been conducted in MG patients. The results of this survey are typical of those seen in other systems. Autoantibodies to AChR were seen in as many as 80–90% of MG patients tested (64); antibody titers varied greatly among individuals and were correlated with disease severity in a relative sense (108). Antireceptor antibodies were directed at multiple dispersed receptor epitopes indicating that this was a polyclonal response (111), and only a small fraction of antireceptor antibodies were directed against the ligand binding site (111–113). The percentage of antibinding site antibodies that were antiidiotypic antibodies was not determined.

Antiidiotypes that bind anti-AChR monoclonal antibodies have been detected in 40% of MG patients (113), and a low but significant level of shared idiotypes appears among antireceptor antibodies of MG patients (115, 114–116). Longitudinal studies of individual patients indicated that the appearance of anti-AChR antiidiotypes was cylical and had a reciprocal relationship to anti-AChR levels (118). These latter results are reminiscent of the regulation of β-adrenergic receptor-binding antiidiotypes discussed previously (53), and most probably represent classical idiotypic regulatory patterns (3, 59). In summary, although excellent evidence points to the role of antireceptor antibodies in the establishment of MG, no direct evidence exists for the presence or functional role of antiidiotypic antibodies that recognize AChR in MG or, in general, for the presence of antireceptor antiidiotypes in any human autoimmune disease.

A Model of Antiidiotypic Autoimmunity

How then do we reconcile these observations with those that demonstrate the spontaneous and pathogenic effects of experimentally induced antiidiotypes? Clearly, at the time of diagnosis most antireceptor antibodies are not either binding-site related or antiidiotypic. We propose the following model to account for these observations.

INDUCTION PHASE According to the principles discussed previously, the production of antiidiotypic antireceptor antibodies could be induced by

virus, by a foreign antigen that contained cross-reactive epitopes with natural ligand-binding determinants, by a modified natural ligand, or by aberrant regulation of antiligand binding site–specific antibodies. Because they are induced through the immune network, we expect these anti-idiotypic antireceptor antibodies to be typically, but not exclusively, IgM and of relatively low affinity.

EXPANSION PHASE The binding of antiidiotopes to receptors on target cells stimulates antireceptor effector responses such as complement fixation and cell lysis, or antibody-dependent cell-mediated cytotoxicity. As a consequence of these events, receptor bound to IgM antiidiotope is processed by adherent cells or B cells and presented to the immune system in a more immunogenic form. This results in the expansion of B- and T-cell clones that recognize alternative or secondary epitopes on the receptor. Evidence for a role of IgM in the trapping of antigen that alters processing to expose secondary epitopes has been recently reported by Harte et al (119) and Lanzavecchia (120).

REGULATORY PHASE The recognition of secondary epitopes on the receptor stimulates the coordinate induction of helper T cells, which amplify this response, and selects for high-affinity IgG antireceptor antibodies. In contrast, the expansion of internal image B and helper T cells is inhibited by the coordinate effects of antiidiotype and antigen on idiotope-positive suppressor T cells. Excellent evidence for the activation of antiidiotope-specific suppression of idiotopic responses has been generated in studies of group A streptococcus and the azobenzenearsonate hapten (121, 122).

PATHOGENIC PHASE The presence of multiple, high-affinity antireceptor antibodies eventually leads to clinical symptoms of disease. The activity of antireceptor antibodies will be controlled in part by the induction of antiidiotypic antibodies, and thus the antireceptor response will cycle. Tissue destruction may lead to a tertiary immune response to a variety of cellular antigens, typical of diseases such as insulin-dependent diabetes mellitus.

This model accounts for all of the observations we have previously presented and discussed. It is also flexible enough to accommodate related observations of autoimmunity such as environmental, genetic, or age linkage. For example, genetic or age associations might be expressed at the clonal level by the restriction, or lack thereof, of internal image anti-idiotopes, while environmental effects could be related to the presence of endemic viruses that bear epitopes related to ligand-binding sites. Finally, this model may be verified experimentally by observing the epitope specificity of antireceptor responses following the administration of monoclonal internal image antiidiotypic antibodies.

CONCLUSION

We have reviewed evidence that antiidiotypic immunoglobulins can be used to identify a variety of receptor proteins. Many of the advantages and limitations of this approach were discussed. Clearly, receptors that have composite binding sites or unusual structural features may be difficult to identify by antiidiotype-based techniques. The characteristics of the various species of antiidiotypic antibodies were also described. We suggest that the majority of antiidiotypic antibodies generated by conventional immunization procedures will not be able to interact with cell-surface receptors, although such antibodies are capable of binding to idiotypic immunoglobulin. The development of reliable techniques to readily induce or enhance those species of antiidiotypic immunoglobulins that mimic ligand binding is clearly needed. To accomplish this, a more thorough understanding of the regulation and function of the various classes of antiidiotypes will be required. In this respect a more complete investigation of the potential relationship of antiidiotypic antibodies to receptor-related autoimmune diseases should prove valuable. We have presented a speculative scheme of the etiology of certain forms of autoimmunity as a framework to define the regulation of autoreactive antiidiotypic antibodies and their contribution to the early and late stages of disease processes.

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CONTENTS

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ANALYSIS OF THE EXPRESSION AND FUNCTION OF CLASS-II MAJOR HISTOCOMPATIBILITY COMPLEX–ENCODED MOLECULES BY DNA-MEDIATED GENE TRANSFER[1]

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INTRODUCTION

General

The major histocompatibility complex (MHC) was first recognized when the genes were mapped that are responsible for acute tissue or tumor graft rejection between members of a species (1). This chromosomal region contains a large number of genes whose products play particularly important roles in the self-nonself discrimination processes of the immune

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system. Recent advances in both in vitro methods of cellular immunologic study and recombinant DNA technology have permitted an explosive increase in our knowledge of the structure of individual MHC genes and their products and of the overall organization of these genes within the MHC. Perhaps equally significant has been the application of DNA-mediated gene transfer (DMGT) to the analysis of structure-function relationships of MHC-gene products. Use of DMGT promises to provide substantial new insight into the actual biochemical bases of immune recognition and regulation, processes highly dependent upon the fine structure of MHC-encoded gene products and the lymphocyte receptors with which they interact. This review focuses on recent work utilizing this approach to identify individual class-II MHC genes and to explore their structure and function. New findings concerning the control of MHC-gene expression, molecular assembly, and the relationship among molecular organization, species polymorphisms, and immune recognition are detailed. Finally, we discuss future applications of these methods and some of the limitations of interpretation of experiments involving these techniques.

Class-II Proteins and Genes

A substantial body of information now exists (2, 3) concerning the structural organization of class-II MHC molecules and genes. Included in the class-II MHC category are the known Ia molecules (I-A and I-E in mice; HLA-DP, DQ, and DR in man), as well as genes homologous to these prototypic family members.

The tissue distribution of class-II molecules is limited to certain cell types and is distinct from that of class-I molecules, such as H2K, D, L or HLA-A, -B, -C. Ia is expressed primarily on B lymphocytes, monocytes, macrophages, dendritic cells, certain epithelial and skin-associated cells, and in species other than the mouse, on activated T lymphocytes. Ia can be induced on a wide variety of Ia^- cells by exposure to γ-interferon (4, 5).

Ia antigens are heterodimeric cell-surface glycoproteins consisting of one heavy (33–34 kd), or α, and one light (28–29 kd), or β, chain (Figure 1). The difference in molecular weights of α and β is due to the presence of an additional N-linked carbohydrate side chain on the α molecule. The two chains are noncovalently associated, and both extend through the cell membrane. Each consists of two extracellular domains of $\sim$96 amino acids. The NH_2-terminal domain of the β chain and the membrane proximal domains of both α and β have internal disulfide bonds, while the NH_2-terminal α_1 domain does not. The membrane proximal domains of both α and β, like that of class-I molecules, show strong homology to immunoglobulin constant-region domains. These regions are followed by

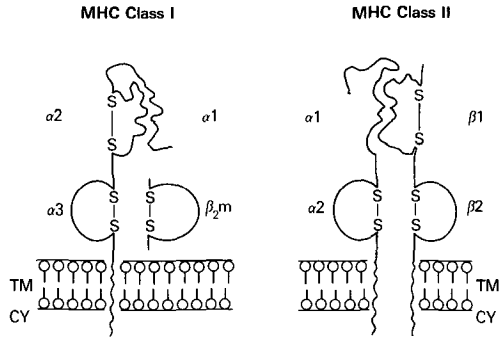

Figure 1 Domain organization of class-I and class-II MHC molecules. Prototypic class-I and class-II molecules are illustrated. The TM (transmembrane) regions are shown spanning the plasma membrane. CY = cytoplasmic tail; α_1, β_1, etc = extracellular domains of the proteins; β_2m = β2 microglobulin; S-S = intradomain disulfide bonds.

membrane-spanning hydrophobic segments and cytoplasmic tails of up to ~12–15 amino acids.

The organization of class-II genes correlates well with the protein structure (Figure 2). Both α and β genes begin with a leader-encoding exon that also contains the codons for 3–6 of the amino-terminal residues of the mature protein. Exons 2 and 3 encode the β_1 or α_1 and β_2 or α_2 domains, respectively. β genes have three additional exons, encoding the TM, CY, and 3′ UT, while α genes have the TM, CY, and the beginning of the 3′ UT on exon 4, with the remainder of the 3′ UT on exon 5.

A large number of allelic forms of the individual class-II genes have been cloned and sequenced (6–31). Comparison of the nucleotide sequences, and especially of the deduced amino acid sequences, revealed that for Aα, Aβ, and Eβ of the mouse, and DPα, DQβ, and DRβ of man, the allelic forms of each gene differ substantially from one another. The allelic polymorphism is concentrated in the amino terminal (β_1 or α_1) domain in each case, and within the domain, in three or four hypervariable regions spread across the domain.

Organization of the MHC

The availability of probes for the different class-II member genes of the MHC has permitted the creation of a molecular map of the murine H-2 complex with respect to these genes and related sequences. Similar studies are in progress in man but remain largely incomplete at this time.

The number and arrangement of class-II genes of the MHC of the mouse have been analyzed in detail for both the H-2^d and H-2^b haplotypes, using cosmid mapping techniques. Steinmetz and his collaborators have identi-

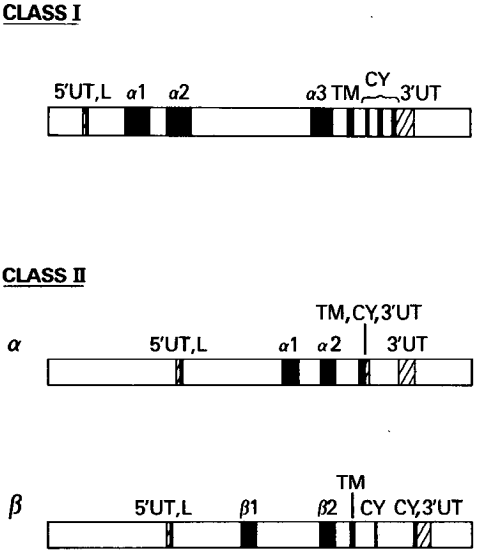

Figure 2 Class-I and class-II MHC gene organization. The intron-exon organizations of a class-I heavy-chain gene, a class-II α-chain gene, and a class-II β-chain gene are shown. 5′ UT = 5′ untranslated region; L = leader sequence; α_1, β_2, etc = exons-encoding the extracellular domains of the various proteins; TM = transmembrane segment; CY = cytoplasmic tail; 3′ UT = 3′ untranslated region.

fied and mapped seven class-II genes or gene sequences in the H-2^d haplotype (32, 33). This includes [Aβ_3-Aβ_2-Aβ-Aα-Eβ-Eβ_2-Eα] in this order, centromere to telomere. Flavell and coworkers have similarly analyzed the H-2^b haplotype, demonstrating the same seven loci found in the H-2^d haplotype, as well as an additional cross-hybridizing sequence termed Eβ_3, located telomeric of Eα (34). The physical distances between the various genes determined by cosmid walking and those reported on earlier maps based on recombination frequency show important differences, due primarily to the occurrence of "hot spots" for recombination. This has been especially well demonstrated for the intron following the second exon of Eβ (32, 35).

Although no complete molecular linkage map of class-II genes has been produced for the HLA region of man, gene counting experiments and cosmid mapping have provided some idea about the number and local organization of these genes in humans (18–31). One DRα, two "DQα" (DQα and DXα), two DPα genes, and one DZα gene have been identified, among which at least one DPα may be a pseudogene. Three DRβ, two DQβ (DQβ and DXβ), two DPβ genes, and one DOβ gene have also been described, with several of these likely to be pseudogenes. The corresponding α and β

genes are always linked in clusters, usually of the β-α, β-α type. The clusters corresponding to DR, DP, and DQ have not been linked to each other.

TECHNICAL CONSIDERATIONS

General

Three main types of gene transfer studies are possible. Transient expression assays evaluate gene transcription or gene product function shortly (a few hours to several days) after DNA introduction into target cells. Stable integration into the chromosomal DNA of the host cell is not required. This method has the advantages of being rapid, of permitting large numbers of constructs, clones, or conditions to be tested in a brief time interval, and of examining expression by a significant (several percent) proportion of target cells, minimizing artifacts due to clonal variation. The method suffers from the need to transfer the genes separately for each experiment, thus introducing variability into the system and precluding the study of homogeneous, permanently available target cells expressing the gene(s) of interest.

Stable transfectants have integrated the introduced DNA into their chromosomes or have an episomal version of the experimental genes maintained in the cell. It takes 1–3 weeks initially to identify clones of transfectants that have acquired the input gene(s), and several additional weeks to isolate clonal or mixed populations with homogeneous expression suitable for many of the desired studies. Target cells are limited to those into which one can introduce DNA successfully at suitable frequency and those able to permit expression at the gene, protein, and if appropriate, membrane level. This method has the advantages of yielding cell lines usable for a continuing series of experiments over a prolonged time period, of permitting easy exchange with other laboratories, and of allowing comparison of cells in which the known gene copy number or level of gene product varies.

The third method involves transfer of DNA into the germline of animals (transgenic animals). This is a difficult procedure available only in a few laboratories. Large series of experiments comparing different genes or gene constructions are not practical, and effects of the integration site on expression are hard to control. However, this is the only good method for evaluating the expression of a gene in a large number of tissues and during ontogeny or differentiation of a given cell lineage. Further, it is the only method available for examining the influence of a single gene product during development and for exploring the way in which the particular tissue(s) in which a given gene product is expressed affects the physiology of the entire individual.

Methods of DNA Introduction

Several methods are available for introducing genes into cells. They can be divided into methods relying on general cellular uptake processes, techniques involving membrane fusion, those in which the cell membrane is traversed by physical means, and those utilizing biological delivery systems. As these procedures have been recently reviewed in detail in this series (36), we will only summarize them briefly here.

CELLULAR UPTAKE The most common means of introducing exogenous DNA into cells is through use of the $CaPO_4$ coprecipitation technique, in which a microcrystalline precipitate of $CaPO_4$ and DNA is formed and applied directly to intact cells (37, 38). A fraction of the cells take up the DNA-containing particles, and a proportion of these incorporate the DNA into their chromosomes. If the site of integration is suitable, the gene of interest has not been disrupted during integration, and the cell is permissive for transcription, then mRNA able to lead to expression of the desired gene product will be produced at some level. Multiple copies of the gene may be integrated, usually as a concatamer (pecalosome) at a single site, though multiple integrations are possible (38). This approach has been used with a variety of target cell types. It seems to work more efficiently with adherent than nonadherent cells, and fibroblastoid or epithelial cells show transformation efficiencies one to several orders of magnitude greater than lymphoid cells. The $CaPO_4$ method is capable of simultaneous introduction of multiple cloned genes, or significant amounts of whole genomic DNA. The DEAE-dextran method, while not involving a physical precipitate, is similar in principle and practice to $CaPO_4$. Both methods give better results if the target cells are osmotically shocked using glycerol, polyethylene glycol, or dimethylsulfoxide, apparently enhancing DNA uptake. Transient transformation efficiencies range from 0.1–50%, while stable transformation occurs at rates of 10^{-5} to 10^{-3}.

MEMBRANE FUSION DNA can be packaged in natural or synthetic lipid bilayers, and fusion between the DNA-containing vehicle and the target cell membrane promoted by various means. The most widely used version of this method involves growing suitable *E. coli* (such as HB101) containing a plasmid bearing the gene of interest. Chloramphenicol amplification leads to high plasmid copy number per bacterial cell. The cell walls are then digested with lysozyme, and the resultant spheroplasts (protoplasts) are fused with the target cells using polyethylene glycol, as for somatic cell hybridization (39, 40). The introduced DNA can then be incorporated into host DNA. This often occurs with a single copy at a single site, but multiple integrations are seen.

The spheroplast method has proven to be somewhat more efficient than $CaPO_4$ for transfection of nonadherent cells, such as lymphocytes and plasma cells, although only in rare circumstances does the transformation efficiency approach that of adherent fibroblastoid cells transfected by the $CaPO_4$ or DEAE-dextran methods. In general, stable transformation rates of 10^{-6}–10^{-4} can be achieved, depending on target cell types.

PHYSICAL METHODS Two distinct physical procedures are in use for DNA introduction. Cells can be microinjected with DNA directly. This method yields a high frequency of cells that have integrated the introduced DNA (41), but the technique is expensive to set up, difficult to carry out, and works readily only on certain cell types, especially large adherent cells with easily visualized nuclei. A more recent approach involves producing transient "holes" in the cell membrane by pulsed electroshock (42). By carrying out this shock with the cells in a solution containing a suitable concentration of linear DNA, high efficiencies of DNA transfection (10^{-4} to $>10^{-3}$) have been reported for cells unsuitable or refractory to other techniques. However, this method has not yet been standardized to the extent that it works readily in a large number of laboratories, although results from many workers suggest it holds great promise.

BIOLOGIC DELIVERY DNA is routinely introduced into eukaryotic cells under natural conditions during viral infection. Attempts have therefore been made to utilize viral vectors to transfer DNA efficiently into target cells. A number of different viruses have been employed, the two most extensively studied of which are vaccinia (43, 44) and retroviruses of the Friend, Moloney, Rauscher group (45–47). Vaccinia cloning vectors containing genes incorporated into suitably modified vaccinia genomes can be readily transmitted to target cells by virus stocks produced using the recombinant DNA. These foreign genes, such as influenza hemagglutinin (48), are expressed at high levels by the infected cells, but such cells eventually die due to the effects of the virus; this approach is thus unsuitable for many studies. Retroviral vectors offer a means of infecting cells with high efficiency, of favoring precise gene integration without disruption, and of enhancing the expression of the transferred gene due to the action of the viral LTR. Numerous groups have designed and tested a whole series of retroviral vectors suitable for murine studies (ecotropic packaging systems) and other species (amphotropic packaging systems). The more recent of these constructs shows great promise in meeting the requirements of transmissibility and gene expression; retroviruses may thus become the method of choice for many gene transfection studies. Under optimal conditions, gene transfer to virtually 100% of a primary target cell population can be achieved by this method.

Selection of Stable Transformants

Although experiments involving transient expression following transfection do not require applying selective pressure on the target cell population, the frequency of stable transformation is too low to be suitable without a means for isolating transfectants with a high likelihood to have integrated the gene of interest correctly. This selection process is most often accomplished by including in the DNA used either a covalent construct involving the experimental gene and a gene conferring drug resistance, or a mixture of such genes. The $CaPO_4$, DEAE-dextran, liposome or RBC-fusion, electroporation, and microinjection methods are all suitable for cotransformation of unlinked DNA, although some workers ligate the DNA before use. By employing a high ratio of test gene to drug resistance gene (10–100 : 1), it has been shown that most cells surviving drug selection contain the gene of interest in addition to the drug-marker gene (38). The spheroplast fusion and retroviral vector methods, although adaptable with difficulty to cotransformation, are most often used with covalent linkage of test and drug selection genes.

The choice of a suitable drug for selection depends on the nature of the target cell, the experimental plan, and in some cases, the ease of producing suitable constructions. Some systems require selection for gene loss (e.g. thymidine kinase deficiency selected by use of BUdR and light), followed by replacement of the missing gene via transfection. Increasingly, however, the method of choice involves dominant selectable markers, such as the aminoglycoside phosphotransferase gene that confers resistance to the neomycin analogue G418 (49), or the xanthine guanine phosphoribosyl transferase *xgpt* gene that permits growth in the presence of mycophenolic acid and xanthine (50).

Clones of transfectants can be selected directly, based on expression of the gene product being studied. This can be accomplished either by fluorescent antibody staining of the transfectant population, followed by preparative flow microfluorimetry (cell sorting) (51, 52), or by use of suitably tagged erythrocytes or latex spheres able to react with antibodies bound to transfectants expressing the desired gene product(s) (53). Clones identified by this latter method are physically isolated and expanded for use.

EXPRESSION OF CLASS-II GENES AND MOLECULES

Gene Expression

EXPRESSION BY Ia$^+$ RECIPIENT CELLS Class-II (Ia) molecules are normally expressed only by a limited number of cell types. Membrane expression is

correlated with mRNA content of cells, indicating primary regulation of Ia expression at the level of gene transcription. Therefore, initial studies on Ia expression following DMGT were carried out using cells showing either constitutive (B lymphomas) or inducible (macrophage tumors) expression of endogenous Ia genes, with the expectation that these cells would be permissive for expression of introduced class-II genes. Using this strategy, several groups have shown that introduction of complete genomic copies of mouse class-II genes into B-lymphoma cells results in high levels of gene transcription and in expression of the protein product of the transfected gene on the cell membrane. Some of the early studies utilized a heterologous system in which mouse genes were introduced into an Ia^+ SV40-transformed hamster B lymphoma (54; P. Jones, personal communication). The class-II α and β chains encoded by the transfected mouse genes could be shown to assemble with each other preferentially, but also with the hamster class-II chains.

More recently, Norcross et al (55) have demonstrated mouse-human interspecies Ia molecules following transfection of A^d_β into an EBV-transformed human lymphoblastoid cell line. The surprising result in this study was that the $A\beta$ chain was associated with DRα, not DQα, even though the latter chain shows the greatest homology to the mouse Aα chain normally found associated with Aβ. Transfection studies have also been carried out using murine B-lymphoma cells. Transfer of the A^k_β gene into the mouse Ia^d-positive Balb/c B-lymphoma M12.4.1 resulted in high levels of membrane A^k_β expression, detected using the anti-I-A^k monoclonal antibody 10.2.16 (56, 57). In these experiments the A^k_β chain was expressed in association with the A^d_α endogenous gene product. Folsom and her collaborators (58) utilized the P388D1 macrophage line as the recipient of an E^b_β genomic clone introduced by spheroplast fusion. The P388D1 cell line is normally Ia^- but expresses high levels of I-A^d and I-E^d following culture for several days in mouse γ-interferon (γ-IFN). After DMGT of E^b_β, the selected P388D1 cells were Ia^- and did not contain either endogenous Ia transcripts or E^b_β transcripts. Exposure of the transfectants to γ-IFN led to expression of $A^d_\beta A^d_\alpha$, $E^d_\beta E^d_\alpha$ and $E^b_\beta E^d_\alpha$ molecules, demonstrating that the introduced E^b_β gene was coordinately regulated with the endogenous class-II genes. These experiments all involved complementing a single introduced class-II β-gene product with an endogenous α chain. More recently, pairs of α and β genes have been introduced into murine B-lymphoma cells. Complete Ia molecules not involving endogenous target cell class-II products have been detected by biochemical analysis and functional studies (59), in accord with the earlier work in hamster cells (54).

The expression of class-II genes following DMGT into Ia^+ recipient cells was not unexpected. Nonetheless, these studies did provide direct demonstration that the genomic class-II DNA clones isolated in various labora-

tories actually encoded the expected gene products and contained sufficient regulatory sequences for efficient expression in a permissive cellular environment. The experiments with P388D1 showing γ-IFN-responsive gene expression further indicated that introduction of class-II genes into abnormal chromosomal locations did not prevent apparently normal inducible control of gene transcription. Despite these important findings, however, the use of these model systems for studying certain aspects of class-II structure-function relationships posed several problems. Because all the available recipient cells expressed endogenous Ia molecules in addition to the transfected gene product, it was difficult to assign any observed gain in serologic or T cell–restriction elements solely to the introduced gene product. The assembly of a variety of Ia molecules following the introduction of both α and β genes, due to complementation with endogenous gene products, prevented clear understanding of which introduced chain(s) actually were involved in any new phenotypic trait. It was also hard to determine the level of expression on the cell membrane of each of the various Ia molecules produced under these circumstances, and this prevented careful quantitative analysis of antigen-presenting function in the context of the transfected gene product(s). Finally, since these target cells were already known to be capable of Ia expression and antigen presentation, no new insight into either control of Ia chain assembly and membrane transport or the physiology of APC function could be derived from these models.

EXPRESSION BY Ia^- RECIPIENT CELLS For all the above reasons as well as for technical considerations, several laboratory groups chose to look at the effects of transferring class-II genes into the Ia^- mouse fibroblastoid L-cell line. L cells lacking the thymidine kinase gene (tk^-) have been derived from the original L-cell line of C3H fibroblasts and are a commonly used recipient cell for a variety of genes. They readily transfect using the $CaPO_4$ method and tk cotransformation at rates approaching 10^{-3}. L cells do not express endogenous class-II genes and remain Ia^- after exposure to γ-IFN or activated T lymphocytes, thus precluding the problems of multiple class-II species that are seen when researchers use B lymphomas or macrophage tumor lines (R. N. Germain, B. Malissen, unpublished observations).

Rabourdin-Combe & Mach (60) were the first to show that L cells could express class-II genes following DMGT. Using cosmid clones that included complete DRβ and DRα genes, these workers cotransfected L cells and selected drug-resistant colonies. Clones expressing a range of membrane DRβDRα molecules were identified, with one clone showing levels similar to EBV-transformed B-lymphoblastoid cells. No expression was seen when either DRβ or DRα containing DNA was introduced separately, and this is

consistent with previous suggestions of a requirement for $\alpha:\beta$ pairing for efficient cell-membrane expression of Ia. Analysis of immunoprecipitated products showed that the DR molecules expressed by transfected L cells were grossly normal biochemically.

These initial results were confirmed for mouse class-II genes by Malissen and colleagues (54, 61) and by Norcross et al (62). In both studies, transfection of α or β genes alone failed to lead to detectable membrane expression, while cotransfection with $A\alpha:A\beta$ pairs derived from the same haplotype (e.g. $A^k_\alpha A^k_\beta$, $A^d_\alpha A^d_\beta$) showed substantial surface expression. Again, electrophoretic gel analysis showed no significant differences in α- or β-chain structure compared to normal lymphoid cell Ia, except that there was little or no Ii in the immune precipitates. However, it is clear that L cells synthesize invariant chain (63), and the failure to see Ii in immunoprecipitated material is not due to the absence of this molecule in L cells.

The ability of transfected L cells to transcribe class-II genes and express Ia in substantial quantities on the cell surface is somewhat unexpected, since no expression of endogenous Ia genes is observed. Although clearly no direct covalent linkage of class-II genes to strong enhancer-promoter combinations is required for expression (62), the use of such regulatory regions indeed enhances the level of class-II expression (61). A certain low but significant amount of class-II-gene transcription of introduced genes occurs in L cells, and selection for cells with high copy number, or integration in sites of active transcription, permits identification of cells with significant levels of Ia. Addition of viral enhancers to class-II genes increases the proportion of transfected cells with high expression by reducing the need for high copy number or special integration sites.

ANALYSIS OF CLASS-II-GENE TRANSCRIPTION The observations detailed above concerning expression of transfected class-II genes in Ia^+ vs Ia^- recipient cells suggest that both *cis*- and *trans*-active elements control Ia gene expression in a partially tissue-specific manner. This is supported by the finding that in γ-IFN inducible cells, transfected class-II genes are coregulated with endogenous genes. DMGT has been used to explore this issue in greater detail.

Gillies et al (64) have utilized a stable transfection model to analyze the region(s) that control efficient transcription of the Eβ class-II gene in murine-B-lymphoma cells. Various segments of 5′ flanking DNA from an E^d_β containing genomic clone were linked to the selectable marker *xgpt*, and the resultant constructs transfected into either B-lymphoma cells, myeloma cells, or L cells. The frequency of drug-resistant colonies was taken to reflect the efficiency of transcription of the *xgpt* gene and to indicate whether the flanking DNA segment showed cell type–specific regulation of transcription. The results of this experiment revealed that *xgpt* constructs containing

5′ flanking DNA from the Eβ clones showed substantially greater ($\geq$50 fold) activity in Ia$^+$ B-lymphoma cells than in either Ia$^-$ myeloma or L cells. The relevant region of DNA mapped to a 2 kb HindIII-BstX1 fragment beginning approximately 600 bp from the initiation of Eβ transcription. Smaller fragments within this 2 kb region were inactive, indicating the possible existence of multiple small regions involved in full activity. The function of this region appeared location and orientation independent, suggesting that a classical enhancer region was involved in the observed effects. A pair of conserved sequences usually found at $\sim$($-$100 to $-$150) 5′ of class-II genes was not included in the active fragment, indicating that these sequences are not required for enhancer function.

Further information on the location of DNA sequences involved in regulation of class-II-gene expression comes from recent analyses of transgenic mice. LeMeur et al (65) have introduced genomic clones of the murine E_α^a gene into (C57B1/6 $\times$ SJL)F_1 mice. These mice fail to transcribe their endogenous Eα genes owing to apparently identical $\sim$650 bp deletions encompassing the promoter region of the E_α^b and E_α^s genes. Injection of DNA containing 2 kb of 5′ flanking DNA and 1.4 kb of 3′ flanking DNA surrounding the complete E_α^a gene gave rise to mice expressing Eα mRNA in precisely the same tissue pattern as the endogenous Aα genes and initiating at the normal site. Similar results were obtained by Yamamura et al (66) and Pinkert et al (67) with somewhat different Eα clones. In contrast, an analysis of transgenic mice containing the E_α^d gene plus only 1.4 kb of 5′ flanking DNA show altered tissue expression confined to the thymus (G. Widera, personal communication). Thus, as for the Eβ gene, the 2 kb region 5′ of the transcriptional start site is critically involved in regulating Eα expression, and within this region there appear to be multiple regulatory elements.

Additional studies using gene transfer to map regions regulating class-II-gene expression are in progress. The data obtained by several groups using transient expression assays and constructs combining fragments of class-II genes with indicator genes such as chloramphenicol acetyl transferase (CAT) indicate a complex set of positive, and perhaps negative regulatory elements with distinct roles in response to γ-IFN and to tissue-specific *trans*-acting factors (D. Mathis; W. Roeder, R. Maki, personal communications). It can be anticipated that substantial definition of the precise regions involved in lymphokine regulation of Ia expression and in control of class-II transcription at different stages of cellular differentiation will be available shortly. In this regard, transcription of both endogenous and transfected class-I genes in L cells is upregulated by γ-IFN, while the transcription of endogenous and transfected class-II genes is not; this observation indicates that cell-specific elements beyond γ-IFN receptors

and DNA target sequences are required for appropriate class-II-gene regulation. Thus, mapping of DNA sequences controlling inducibility in cells such as macrophages will only provide part of the picture, and identification of *trans*-acting factors and/or negative regulatory regions active in uninducible tissue types will be required for a more complete understanding of this issue.

Control of Ia Expression at the Protein Level

As mentioned above, detection of Ia on the cell membrane of transfected L cells required introduction of both α and β genes. Transfer of either α or β genes independently did not lead to surface expression as assessed using a variety of monoclonal antibodies. These results agreed with those obtained using Ia^+ recipient cells, in that transfer of single α or β encoding genes only led to expression through pairing with endogenous complementary class-II-gene products. One important caveat to this view that $\alpha:\beta$ heterodimers are required for surface expression should be noted—most of the reagents used for detection of Ia molecules have not been shown to react with isolated α or β chains, which presumably have a significantly different configuration when not assembled in a heterodimer. Thus, surface expression of individual chains might be missed using these reagents. However, additional experiments are also consistent with a lack of surface expression of free α or β chains.

In one study, McCluskey et al (68) compared surface expression of the A^k_β gene product by L cells to the membrane expression of a chimeric class-II:class-I gene product. This latter molecule was comprised of the $A^k_{\beta 1}$ domain covalently linked to the $\alpha 3$, TM, and CY portions of the class-I D^d molecule. Following DMGT into L cells, this chimeric gene led to the expression of a unique cell-surface molecule detectable with both anti-A^k_β (e.g. 10.2.16) or anti-$\alpha 3(D^d)$ monoclonal antibodies. The same anti-Ia antibodies failed to detect surface molecules in L cells receiving the native A^k_β gene and known to contain high levels of A^k_β mRNA. This pair of cells was also analyzed using a rabbit heteroantiserum prepared against purified $I\text{-}A^k$ molecules. This reagent had been shown to immunoprecipitate free $A\beta$ chains from a reticulocyte lysate in vitro translation system (69) and to detect both $A\alpha$ and $A\beta$ polypeptides in western blots (A. Sant, R. N. Germain, unpublished observations). Again, the cells transfected with the chimeric gene construct stained, while those containing the native A^k_β gene did not. Although one cannot formally exclude the possibility that these results still reflect the use of unsuitable serologic reagents, they add credence to the view that isolated α and β proteins do not reach the cell surface efficiently and further imply that the $A\beta_1$ domain per se does not prevent surface expression.

The postulated role of the Ii (invariant) chain in membrane expression of class-II molecules has been explored using DMGT. Two groups have inserted class-II sequences into SV-40-based cDNA-expression vectors suitable for use in COS cells. These monkey fibroblasts have been transformed with defective SV-40 virus, so that they maintain expression of the SV-40 large T antigen required for replication of DNA containing SV-40 origins (70). Introduction of plasmids containing such SV-40 origins leads to extensive replication. If suitable (e.g. SV-40 early or late region) enhancer-promoters are present upstream of the gene of interest, high levels of transcription also are seen. Employing this system, these groups have found that cotransfer of $A\alpha : A\beta$, $E\alpha : E\beta$, or $DR\alpha : DR\beta$ gene pairs into COS cells leads to surface expression of I-A, I-E, or DR molecules, respectively (J. Miller, J. Tou, A. Sant, R. N. Germain; R. Sekaly, E. Long; in preparation). This surface expression of Ia occurs even though COS cells lack detectable invariant chain (Ii) transcripts, and this implies that invariant chain is not required for transport of Ia to the cell membrane. However, due to the nature of the COS cell system, it is not possible to determine if Ii normally increases the efficiency of Ia transport.

L cells have recently been used to reexplore the issues of allelic control of $\alpha : \beta$ chain pairing and restrictions on cross-isotype $\alpha : \beta$ assembly. Based on the initial findings of Fathman & Kimoto (71) and of Silver et al (72), it is generally accepted that Ia^+ cells from heterozygous individuals contain a mixture of Ia molecules derived from the free assortment of allelic α and β chains of a single isotype in all possible combinations. Thus, in $(H\text{-}2^b \times H\text{-}2^k)F_1$ mice, one would find $A^b_\beta A^b_\alpha$, $A^b_\beta A^k_\alpha$, $A^k_\beta A^b_\alpha$, and $A^k_\beta A^k_\alpha$ heterodimers in approximately equivalent proportions. Such α- and β-chain mixing, while occurring within an isotype, did not seem to occur between α and β chains of distinct isotype, as mixed molecules such as $A\beta : E\alpha$ had never been demonstrated. These results were consistent with control of $\alpha : \beta$ assembly lying within the isotypically conserved COOH-terminal (β_2 or α_2, TM, CY) portions of the molecules, and with little role for the NH_2-terminal polymorphic portions of the chains in this process. There are, however, several reports in the literature inconsistent with this view of Ia assembly : expression. For instance, McNicholas et al (73) found an 8–10-fold preference of $E^u_\beta : E^u_\alpha$ assembly over $E^k_\beta : E^u_\alpha$ assembly in cells of the $(B10.A(4R) \times B10.PL)F_1$ mouse, and Conrad et al (74) showed lesser, but significant, allele-specific variation in expression of $E^{b,d,k}_\beta$ with E^d_α. These data indicate an important role for the highly polymorphic $E\beta_1$ domain in I-E expression. In addition, Lerner et al (75) commented on the possible precipitation of $A\alpha$ together with $E\beta$ and $E\alpha$ molecules, implying the existence of $E\beta : A\alpha$ isotypically mixed dimers. However, for the most part,

the I-E preferential pairing results were taken as unusual cases, and the $E\beta$: $A\alpha$ possibility never directly demonstrated.

A view of Ia-chain pairing much closer to the latter "unusual" results, and quite distinct from the generally accepted model, has emerged from L cell–transfectant data (76). During attempts to develop cell lines expressing only F_1-type Ia molecules (e.g. $A^k_\beta A^d_\alpha$), it was noted that while haplotype-matched $A\alpha$: $A\beta$ pairs gave a high proportion of primary L-cell transfectants expressing significant levels of membrane Ia, cotransfection of haplotype-mismatched pairs gave little or no expression. This was true even though the DNAs employed for the matched or mismatched gene pairs were identical, and despite the presence of readily detectable $A\alpha$ and $A\beta$ mRNA in the nonexpressing cells. Further experiments revealed that for genes of the b, d, and k haplotypes, *cis*-chromosomal $\alpha:\beta$ pairs (e.g. $A^k_\beta A^k_\alpha$) always gave better expression than *trans*-pairs (e.g. $A^b_\beta A^k_\alpha$); experiments also indicated that the level of expression of the latter varied over a wide range, depending on the particular allelic forms of α and β employed. Careful titration studies, the use of a large panel of monoclonal antibodies, and the use of the rabbit heteroantiserum to I-A discussed above, all indicated that these findings were not an artifact of serology, but truly reflected a different efficiency of $\alpha:\beta$ assembly and/or membrane expression for the various allelic forms of the genes. Particularly striking was the finding that irrespective of haplotype, $\alpha:\beta$ pairs normally coinherited due to chromosomal linkage seemed to express best. Also, $A^k_\beta A^b_\alpha$ and $A^b_\beta A^k_\alpha$ molecules, the basis for suggesting "free pairing," are in fact the best expressed of the haplotype-mismatched mixes, while $A^d_\beta A^k_\alpha$ has in fact not yet been detected. $A^k_\beta A^d_\alpha$ is intermediate in efficiency, and the ease of seeing $A^k_\beta A^d_\alpha$ after transfection of B-lymphoma cells appears to be related to the very high amount of A^d_α in such cells, which permits even inefficient pairing to give significant surface expression of $A^k_\beta A^d_\alpha$.

N. Braunstein and R. N. Germain have gone on to map the region of the $A\beta$ molecule controlling this preferential pairing (manuscript in preparation). We constructed a series of recombinant $A\beta$ genes involving the b, d, and k alleles, in which the entire $A\beta_1$ domain was exchanged with the β_2, TM, and CY regions of a different allelic gene, or in which the NH_2-terminal half of $A\beta_1$ was assembled with the COOH-terminal portion of $A\beta_1$, and various $\beta 2$, TM, and CY regions. These "domain or hemi-domain shuffled" $A\beta$ genes were cotransfected independently with $A^{b,d,\text{ or }k}_\alpha$ into L cells; then the number of Ia^+ transfectants and level of Ia expression were monitored by flow microfluorimetry. The results clearly showed that the most important portion of $A\beta$ with respect to $\alpha:\beta$ pairing was the NH_2-terminal half of $A\beta_1$, in that molecules containing this region from a given allele

expressed best with the *cis*-matched Aα, and at levels similar to wild-type Aβ, irrespective of the origin of the remainder of the Aβ chain.

Equally unexpected findings arose when isotype-different α : β pairs were cotransfected into L cells (126). Introduction of A_β^k and $E_\alpha^{a/k}$ gave no surface Ia detectable with either anti-Aβ or anti-Eα antibodies. However, A_β^d did pair with Eα to produce surface molecules reactive with MKD6 (anti-I-A_β^d) and 14.4.S (anti-I-Eα) antibodies. Immunoprecipitation studies showed that these chains existed as noncovalently associated heterodimers. Use of recombinant Aβ genes has shown the differential ability of A_β^k vs A_β^d to pair with Eα to map to the $A_{\beta 1}$ domain, as expected, based on the predominance of allelic variation in this domain (A. Sant, R. N. Germain, unpublished observations). These results are consistent with those mentioned earlier showing A_β^d assembly with DRα, the human equivalent of Eα (55).

These findings support the view that Aα and Aβ genes located on the same chromosome (*cis*-pairs) actually coevolve for best "fit," such that *cis*-pairs form more efficiently than *trans*-pairs. This means that in heterozygous situations, a variable and generally low level of *trans*-paired Ia molecules will be found on the cell membrane, and that most immune responses will occur in the context of the *cis*-paired Ia molecules. Further, these data and the data on cross-isotype molecules indicate that control of α : β pairing is not the province solely of the conserved COOH-terminal portions of the molecules but, rather, is strongly influenced by the highly polymorphic amino termini. This may reflect the role of these regions in pairing of nascent chains during synthesis, or it may result from requirements of interactive folding in the assembly of the complete molecule. Additional studies are clearly needed to determine what if any role is played by the more isotypically conserved portions of the α and β chains in controlling heterodimer formation. Reexamination of normal cells for the existence of cross-isotypic molecules is clearly necessary, as is a series of competition experiments in transfected L cells to determine whether these previously unobserved molecules can form in the presence of the "appropriate" partner chains. Finally, it should be pointed out that such unexpected pairings can explain certain previously reported results (77). The A.TRF5 mouse possesses an MHC containing an intra-I-region recombination event between the f and k haplotypes. This mouse does not produce Eβ molecules but does have a low level of E_α^k molecules on the cell membrane. One explanation of this result that does not require postulating free Eα on the membrane is the possible existence of $A_\beta^f E_\alpha^k$ dimers in these cells. Such pairs may be weakly bound together and easily dissociated by monoclonal antibodies during immunoprecipitation; this would account for the lack of observed coprecipitation. The expression of Eα on the cell membrane of previously negative Eα transfectants following supertransfec-

tion with A^f_β would be consistent with this possibility, and experiments testing this prediction are underway.

STRUCTURE-FUNCTION ANALYSIS OF CLASS-II MOLECULES

General

One of the primary goals of DMGT studies using class-II genes is to determine how the structure of Ia molecules relates to their immunological functions—in particular, how polymorphic variation accounts for the observed specificity of Ia-restricted antigen recognition by T lymphocytes, for Ir-gene effects, and for allorecognition. The issue of whether class-II molecules also subserve a receptor function—triggering the cells bearing them to produce monokines (78) or to initiate differentiation when bound by T-cell receptors (79)—is approachable with this technology. Further, transfection of class-II genes permits evaluation of the physiology of antigen-presenting cell function by exploration of the features required in addition to Ia expression for a cell to present native antigens to T cells. Finally, quantitative analysis of the relationship between Ia expression and T-cell responses, and examination of possible interactions with Ia of nonclonally distributed T cell surface molecules such as L3T4, can be carried out using this model.

Antigen Presenting Cell (APC) Function of Ia-Transfectants

CELL TYPES Three distinct cell types have been transfected with class-II genes and tested for their ability to present antigen to, or to be recognized by, T lymphocytes—murine B lymphomas (M12.4.1, A20-2J), a murine macrophage tumor (P388D1), and L cells. In all cases, transfectants expressing levels of Ia within a few-fold of the endogenous level of Ia expression by the B-lymphoma cells have been shown to present antigen to T-cell hybridomas (54, 56–59, 62). Ia^+ B lymphomas and L cells tested with T-cell clones and primary T cells stimulate these as well (57, 62, 80, 81).

ANTIGEN-PROCESSING The ability of these various cells to act as antigen presenting cells (APC) for T hybridomas is not surprising in light of the ability of Ia-containing planar membranes to do so (82). However, the planar membranes require the use of peptide antigens ("preprocessed" antigens), while native antigen can be presented by the transfected cells. B-lymphoma cells had previously been shown to process antigen (83), but L cells were not necessarily expected to be capable of this. Direct examination of L-cell antigen processing has revealed that these fibroblastoid cells can

carry out the steps necessary to convert native antigen to a form recognizable by T cells (80). This analysis involved demonstrating (*a*) that Ia-transfected L cells can present native antigen to T cells previously shown to respond only to an altered form of the original immunogen, and (*b*) that after interaction with native antigen for a suitable time period (several hours), Ia-bearing L cells could be fixed with aldehyde and would still stimulate T cells, while fixation prior to antigen pulsing prevented T-cell activation. These findings suggest that antigen processing is based on biochemical processes more widespread than just among lymphoreticular elements. It is likely that the receptor-mediated endosome cycling system is involved, a supposition consistent with the effects of lysosomotropic agents on processing (84). Whether cells like L cells are as efficient as normal APC in this activity is unclear. Recently, Shastri et al (81) have found that certain I-A^k restricted, hen egg lysozyme (HEL)–specific T-cell clones respond only to a peptide fragment of HEL, but not to native HEL, when using I-A^k-expressing L-cell transfectants as APC. This did not seem to be a simple quantitative defect and may indicate some minor qualitative differences as well in antigen processing by L cells. Nonetheless, L cells clearly carry out this critical step in antigen presentation quite well for a wide variety of antigens.

IMPORTANCE OF QUANTITATIVE VARIATIONS IN Ia EXPRESSION Numerous studies have shown the critical importance of level of Ia expression on the efficiency of antigen-presentation to T cells (85–87). These findings bear on functional studies of transfected Ia genes in two major ways. First, the ability of DMGT to generate cells with defined levels of surface Ia, and to do so using cells not responsive to Ia-regulatory signals, such as γ-IFN (e.g. L cells), provides a unique means of examining quantitative variation in T-cell activation as a function of Ia density per cell or per assay culture. Second, the recognized importance of variation in Ia levels on T-cell triggering makes it imperative that all structure-function experiments using variant Ia molecules be carried out using cells with defined levels of Ia expression known to be at or above the amount required for stimulation of the test T cell using native Ia.

Transfected L cells displaying the same Ia molecules but varying over 15-fold in membrane expression have been isolated by picking random clones or by selective cell sorting. Use of these cells in careful dose-response titrations confirms earlier findings of a dependent relationship between (Ia) and (antigen) needed to achieve a given T-cell response such that: *Response* = k [(Ia) × (Antigen)] over a given range of Ia density and antigen concentration (80). Additional studies are needed to determine if there is a cutoff level of Ia density per cell below which increasing antigen

levels cannot compensate, or whether the product relationship is a continuum. Similarly, high level effects have not been explored extensively yet.

With respect to the second issue, analysis has been done of T-cell responses to transfected L cells that bear widely disparate levels of surface Ia. In many cases the apparent inability of L cells to present antigen to a given T cell is strictly a reflection of a quantitative, not qualitative, defect; the use of a transfectant bearing a higher level of the same Ia molecule leads to good stimulation (R. Lechler, R. N. Germain, unpublished observations). Such findings indicate that great caution should be exercised in interpreting negative data using transfectants bearing modified Ia molecules. Only when extensive controls for quantitative variation in Ia levels between stimulatory and nonstimulatory cells are employed can the lack of presentation to a particular T cell be ascribed to a qualitative effect of the mutation being studied.

T CELL–ACCESSORY MOLECULES T-cell interaction with accessory cells involves not only recognition of antigen plus Ia by the clonotypic receptor, but the poorly understood function of several nonclonally distributed T cell–surface molecules. The two of major concern for studies of Ia-transfected APC are L3T4 (T4 in man) and LFA-1.

LFA-1 is found on a broad array of cells of hematopoietic origins and consists of one α chain of 170 kd and one β chain of 90 kd. The β chain is shared with a family of related molecules possessing unique α chains (88). Studies on in vitro T-cell model systems suggest that LFA-1 serves as a "cell-adhesion" molecule, facilitating T cell–APC or target cell interaction in an antigen-nonspecific manner (89). Antibody to LFA-1 strongly inhibits virtually every T-cell activity dependent on interaction with a second cell type of hematopoietic origin. However, antimouse LFA-1 has no effect on murine–T cell responses to Ia-transfected L-cell APC when that cell expresses the same Ia molecules as B-lymphoma cells whose interaction with the T cells is blocked under the same conditions (80, 90). This same result is observed with several different antimouse LFA-1 reagents to distinct epitopes (91) and has led to the suggestion that L cells lack the putative target structure for LFA-1 binding. This interpretation does not explain, however, why the same T cell is blocked by anti-LFA-1 from responding to a B lymphoma, yet is able to respond to an L cell lacking the target structure and, hence, even more deficient in LFA-1-mediated adhesion. This issue is made even more confusing by the recent finding that human T cells recognizing class-I (HLA) molecules on transfected L cells can be inhibited by antihuman LFA-1 antibody (92). One possible explanation of these findings is that (*a*) some other L-cell molecule

substitutes for the adhesion normally mediated by LFA-1 (e.g. fibronectin), and (*b*) the human LFA-1 target is different from that of mouse LFA-1 and is present on L cells (perhaps a carbohydrate). Further studies are needed to resolve this point, but this difference in LFA-1 function should be kept in mind in evaluating structure-function studies using L-cell transfectants.

L3T4 is a surface glycoprotein found primarily on helper-inducer, Ia-restricted T lymphocytes (93). This concordance of L3T4 expression and Ia restriction has led to the proposal that L3T4 binds to Ia via a monomorphic epitope and facilitates T cell–APC interaction (94–96). Experiments employing a class I (D^d)–specific, but $L3T4^+$, T hybridoma were taken to be consistent with this model (97), but differences in amounts of D^d and Ia on the various cells used in these experiments made clear comparisons difficult. L-cell transfectants have been used to reexplore this issue in two ways. Greenstein et al (98) utilized a set of transfected L cells bearing either D^d alone or D^d plus $I\text{-}A^k$ as stimulator cells for the class-I specific hybridoma. They found that anti-L3T4 antibody only inhibited the activation of this T cell when two conditions were met: (*a*) the amount of D^d in the system was suboptimal, and (*b*) $I\text{-}A^k$ was present on the D^d-transfected L cell. These data are consistent with the earlier results and support the view that L3T4 acts by augmenting cell interactions via recognition of Ia.

A second approach to this question was taken by Golding et al (99). These workers were able to produce $L3T4^+$ class II–specific CTL lines capable of lysing transfected L cells bearing either conventional $I\text{-}A^k$ molecules or the chimeric [$A^k_{\beta 1}$-$\alpha 3$, TM, CY (D^d)] molecule mentioned above. They then demonstrated that αL3T4 blocked the lysis of transfectants bearing the chimeric molecule. These findings were taken to indicate either that (*a*) L3T4 does not require the α or the isotypically conserved $\alpha 2$, $\beta 2$, TM, or CY portions of Ia for interaction and can do so solely via recognition of the $A\beta_1$ domain, or that (*b*) L3T4 does not bind to Ia, but anti-L3T4 interferes directly with T-cell activation, an interpretation made less likely by the work of Greenstein et al. Although these two studies put important constraints on any interpretation of L3T4 function, they do not fully explain all findings on anti-L3T4 inhibition of T-cell activation. An important next step will be to combine these two approaches to determine if the chimeric MHC gene can substitute for intact Ia in conferring anti-L3T4 sensitivity on the response of the D^d specific hybridoma. If so, further mapping of the L3T4 target structure will be feasible. A more direct approach involving actual binding studies will be possible with the recent cloning of the L3T4 gene.

Apart from information gained about the mechanism of L3T4 action, it is important to point out that these studies show clear involvement of L3T4 in T-cell responses to Ia transfectants. This means that different patterns of

response to Ia variants may be due not to changes affecting recognition by the clonotypic receptor but to changes influencing L3T4 function. As it is apparent that the reactivity patterns of different T cells are affected in markedly distinct ways by L3T4 function, one needs to evaluate the importance of L3T4 in the particular T cell–APC combination being examined before deciding that a mutation in Ia has affected recognition by the MHC-restricted antigen receptor.

Antibody and T-Cell Recognition of Ia

STUDIES USING INTACT GENES Transfectants prepared using complete wild-type α and/or β genes have been analyzed using extensive panels of monoclonal antibodies and T-cell clones or hybridomas. B-lymphoma cells, macrophage tumors, or L cells expressing haplotype-matched pairs of $\alpha:\beta$ chains show precisely the same serologic and T cell–stimulatory activity as cells bearing the same Ia molecules based on expression of endogenous genes (54, 56–62). These results imply that any cell type–specific posttranslational differences in protein processing or glycosylation that may exist among these cells have little or no effect on the immunologically relevant structural features of Ia.

Transfer of intact class-II human genes into L cells has proved particularly useful in that the resulting transfectants permit unequivocal assignment of previously defined serologic epitopes to known $\alpha:\beta$ pairs from a given haplotype. For example, we now know that transfectants prepared using the DRα gene together with a DRβ gene from a DR4, w6 heterozygous cell and determined by RFLP analyses to be the DRw6 β-gene showed reactivity with antibodies to the supratypic determinant MT2. These findings established that this epitope is dependent on DRβ (100). Other studies have investigated cells prepared using DP $\alpha:\beta$ pairs and demonstrated reactivity of such transfectants with both certain antibodies and cytotoxic T cells (101, 102). Extensions of these early investigations should greatly increase our understanding of the relationships among the specificities defined by tissue typing and the actual human class-II-gene products expressed by various haplotypes.

Ia transfectants prepared by introducing only a single gene into Ia^+ B-lymphoma cells to generate haplotype-mismatched $\alpha:\beta$ pairs (56, 57, 103, 104), or by cotransfection of L cells with such "F1" type $\alpha:\beta$ pairs (N. Braunstein, R. N. Germain, in preparation), are useful for determining whether one or the other or both α and β chains of a parental (haplotype-matched) Ia molecule are required for the formation of a particular serologic or T cell–restriction epitope. Although cross-reactivity of monoclonal antibodies with endogenous Ia of B-lymphoma cells decreases the utility of these cells for such an analysis, the results from both systems are

concordant to date. Many monoclonal antibodies appear to require the polymorphic residues of only one or the other parent chain to produce the relevant epitope. Among more than 30 antibodies tested only a small number reacted with a site involving contributions of both α and β chain allelic variation (N. Braunstein, R. Germain, in preparation). It should be kept in mind that these conclusions apply only to Ia molecules taken as a whole, not to the individual α or β chains in isolation. That is, these studies have not addressed whether the determinant is formed exclusively by α or β by itself or requires appropriate overall $\alpha:\beta$ interaction and specific polymorphic residues to create the site. Data suggesting that the latter is usually the case are discussed in the following section.

As far as T-cell recognition is concerned, a general rule (albeit one with a few interesting exceptions) has emerged from use of haplotype-mismatched, Ia-expressing transfectants. Reports of F_1-specific T cells previously established that at least some T cells required contributions of both α and β polymorphic residues for the creation of the restriction site seen (105). These studies did not address the issue of whether this was true only of rare cells selected for this phenotype or of most T cells. In a panel of 20 T hybridomas analyzed by Lechler et al (106), using Ia-transfected L cells bearing all expressed pairs of $A_\beta^{b,d,k}$ and $A_\alpha^{b,d,k}$, only 1 cell was able to respond to an Ia molecule containing the original restricting element Aβ chain and an allelic α chain, and only 1 cell was able to respond to an Ia molecule containing the original α chain and an allelic β chain. Cohn et al (103) also found only a few T cells from an extensive panel that were able to respond to an $\alpha:\beta$ pair other than the one to which they were originally restricted, and these T cells were alloreactive, not antigen specific. It is not yet known if the few T cells that respond to inappropriate $\alpha:\beta$ pairs do so with the same efficiency as they do to the original Ia molecule, or if, in fact, they represent very high avidity cells able to show some activation even though a substantial amount of binding energy is lost due to the change in $\alpha:\beta$ pairing. In general, then, in contrast to antibody recognition, it appears that most T cells depend on allele-specific contributions from both α and β chains for the structural features of Ia responsible for restricted antigen recognition.

STUDIES USING RECOMBINANT MOLECULES The next level of dissection of Ia structure-function involves resolution of the portions of the α or the β chains contributing to serologic or T cell-recognition. This has been approached by several groups through the use of "exon-shuffling" carried out in a manner similar to that described for class-I genes (107, 108). For the most part, regions of individual genes have been exchanged for the corresponding portion of an allelic form of the same gene. In some cases, however, variants have been created by combining pieces of unrelated genes.

The initial reports concerning exon-shuffled class-II genes involved experiments in which the first and second exons encoding the β_1 domain of E^b_β or A^k_β, were exchanged for the corresponding portions of the E^d_β or A^d_β genes, respectively (109, 110). The E^b_β-E^d_β recombinant was introduced into P388D1 (Ia^d) cells and induced with γ-IFN. The A^k_β-A^d_β gene was contransfected into L cells with A^k_α. Following expression of the recombinant gene product together with the appropriate α chain on the cell surface, the cells were stained with monoclonal anti-Ia antibodies or used as APC. In both cases, the recombinant gene products behaved in a way identical to that of the donor of the β_1 domain with respect to antibody binding and T-cell recognition. These results were consistent with the known concentration of allelic variation in the β_1 domain and with the importance of such variation in generating serologic and restriction epitopes. The data with the Eβ gene were also consistent with the previous documentation of identical T-cell responses to cells from mice with naturally recombinant Eβ genes such as B10.A(5R), which has an exchange of $E^b_{\beta 1}$ for $E^k_{\beta 1}$ (35) and acts like parental E^b_β.

A much more extensive series of recombinant genes has been produced by exchanging the first and second exons of $A^{b,d,\text{ or }k}_\beta$ among all three genes, or by exchanging the first exon and 5′ half of the second exon with the 3′ half of the second exon and the remainder of the Aβ gene for the same three alleles, to produce a completely permuted set of Aβ gene segments. These were cotransfected individually with either $A^{b,d,\text{ or }k}_\alpha$ into L cells, and transfectants expressing suitable levels of membrane Ia derived from those combinations that showed detectable assembly (see above). When stained with a large panel of monoclonal anti-I-A antibodies that had previously showed essentially exclusive dependence on α- or on β-chain polymorphism, an unexpected result was obtained (N. Braunstein, R. Germain, in preparation). Although for each antibody it was possible to identify either an Aα-gene product or a region of the appropriate Aβ-gene product required for antibody recognition, combinations of chains possessing such an α-chain or β-chain region that failed to be seen by the antibody were noted. These negative molecules followed a consistent pattern. In each case, they involved a pair of α : β chains with a mismatch in the NH_2-terminal β_1 segment critical to α : β pairing, as described in an earlier section. Thus, for example, the B17-123 (anti-I-$A^{b,d}$) antibody required the COOH-terminal half of $A^{b\text{ or }d}_\beta$ for binding. However, a molecule involving an NH_2-terminal $A^k_{\beta 1}$: COOH-terminal $A^d_{\beta 1}$: [Aβ_2, TM, CY^d] chain paired with A^d_α was not recognized by B17-123, while the same β chain paired with A^k_α was recognized. This was true even though the antibody does not react with I-A^k. The most reasonable explanation for such data is that the α and β chains interact strongly and intimately with one another, and this influences chain folding and, hence, epitope formation. This is consistent with the require-

ment, noted previously, for both the proper α and β chain for T-cell recognition.

Use of this panel of recombinant gene transfectants as APC with a large number of responding T cells gave largely concordant results (106). A large number of T cells were unresponsive to recombinant β chains (with respect to the T cell's original restricting element). Of those T cells that tolerated any changes, most required the proper NH_2-terminal segment involved in the chain-pairing process but could accept some, but not other, substitutions in the COOH-terminal part of $A\beta_1$. For one of the two cells able to accept changes in the NH_2-terminal part of $A\beta_1$, careful dose-response measurements showed significant (>10 fold) shifts in dose-response curves using these variant $A\beta$ chains. Thus, changes in Ia structure affecting $\alpha:\beta$ chain pairing and serologic recognition have important effects on T-cell recognition as well. These findings have important implications for the physical interpretation of these studies, and those involving more limited mutations. The evidence for combinatorial, or more likely conformational epitopes resulting from extensive $\alpha:\beta$ chain interaction implies that any change introduced into the polymorphic domain of Ia may act by altering the conformation of Ia elsewhere in the molecule, at the actual site of T-cell recognition or antigen interaction. Thus, no direct correlation of Ia mutation site and the site(s) of binding to the T-cell receptor or antigen can be made. Such studies can only identify residues that contribute in an important but undefined manner to the structural bases of Ia function. Some other more direct (e.g. crystallographic) determination of the structure of Ia is needed to interpret such mutagenesis data physically. This is not to say that mutagenesis is not a useful tool. The cataloging of permissive and nonpermissive mutations will eventually prove valuable as more precise structural data emerge. More immediately, such studies can still approach the issue of whether distinct residues or sets of residues determine T cell–receptor recognition of Ia independently from any postulated binding of antigen to Ia (111–115), whether or not it is possible to say if such residues are themselves the physical site of such events.

Several additional points emerge from this analysis. Different T cells tolerate changes in different segments of $A\beta$, or mixing of various α's and β's, to different extents. These variations do not seem directly correlated with L3T4 expression or function, raising the possibility suggested earlier by antibody blocking studies (116–118) that there are multiple regions on a single Ia molecule used by T cells for restricted antigen recognition, and that no single pattern of sites for receptor binding or antigen interaction may exist. Alternatively, this apparent complexity may still reflect quantitative variation in receptor avidity, giving the appearance of different qualitative recognition patterns. Careful quantitative tests may help clarify

this point. In addition, the data make clear that comparison between only two allelic forms of a gene may be misleading. That is, although a region may be considered uninvolved because one residue or chain segment can be substituted for another without detriment, this may merely reflect the fact that the changes made are conservative or permissive. A different substitution at the same position might significantly alter function. This leads to the view that failure to see a change in response due to a change in Ia between two given alleles should be conservatively interpreted as indicating that the site in question is not responsible directly for the distinction between those particular haplotypes, without being considered informative as to whether the site plays a role in the overall recognition process.

In addition to these experiments involving exchanges between homologous genes, another involved analysis of one molecule comprised of the $A^k_{\beta 1}$ domain linked to the $\alpha 3$, TM, CY portions of the class-I D^d molecule that was expressed on L cells. McCluskey et al (68) demonstrated that of six monoclonal anti-I-A^k antibodies that showed indistinguishable reactivities with interallelic Aβ shuffled genes containing $A^k_{\beta 1}$ paired with A^k_α, only one antibody still reacted equivalently with the isolated $A^k_{\beta 1}$ domain of this chimeric molecule. The others showed weak or no reactivity, indicating that although the $A^k_{\beta 1}$ domain was required for the generation of the relevant epitopes, additional contributions from $A\beta_2$ and/or Aα were needed to produce the native structure. These results again point out that direct physical interpretation of data obtained by recombination or mutagenesis is not possible.

A second, perhaps more surprising, result involving this class II–class I molecule was the finding that CTL raised to conventional I-A^k molecules could recognize L cells bearing only $A^k_{\beta 1}$ and lyse such targets (99). This seems to conflict directly with the results, discussed above, of testing antigen-specific hybridomas for α:β pairing requirements. Consistent with the previous findings, however, the L cells bearing the chimeric molecule fail to act as APC for the I-A^k-restricted T hybridomas requiring A^k_β:A^k_α pairs (R. Lechler, J. McCluskey, D. Margulies, R. Germain, unpublished observations). CTL from early cycles of stimulation only lyse I-A^k, and not $A^k_{\beta 1}$-bearing L cells, while CTL from later cycles lyse both types of transfectants; this implies a change in phenotype with time in culture. Although more experiments are needed to explain these results fully, one possibility is that a small number of very high avidity cells are selected by repeated in vitro stimulation. Further, the effective concentration of ligand on the targets of these alloreactive CTL is likely to be significantly higher than on Ia-bearing L cells in the presence of exogenous antigen (119), which is the situation when testing antigen-specific hybridomas. Finally, one assay involves CTL function, the other IL-2 release. The combination of these

factors may permit a detectable "response" to $A^k_{\beta 1}$ in the absence of Aα, even though the actual avidity of interaction is substantially reduced compared to an intact I-A molecule. This would then represent an extreme case of what was seen with the antigen-specific T cells using recombinant Aβ genes or mismatched α:β pairs, rather than a different mode of T cell–Ia recognition.

STUDIES USING GENE PRODUCTS WITH LIMITED MUTATIONS To determine the involvement of individual amino acids in generation of serologic epitopes or T-cell-restriction sites on class-II molecules, several groups have begun to analyze the properties of transfectants prepared with class-II genes subjected to site-directed mutagenesis. Alternatively, immunoselected mutants showing limited but reproducible functional differences from wild-type have been analyzed by cloning their class-II genes and carrying out nucleotide sequencing to localize the mutation.

Cohn et al (103) have independently introduced three A^k_β residues in place of the A^b_β residue at the same position, using oligonucleotide-directed mutagenesis. The changes involve Tyr → His at position 9, Gly → Pro at position 13, and Pro, Gln, Ile → -Tyr- at positions 65–67 (where A^k_β is deleted relative to A^b_β). These new Aβ genes were cotransfected with or without A^b_α into Ia^d-positive B-lymphoma cells, and the resultant transfectants were used in panel tests with monoclonal anti-I-A antibodies and I-A^b-specific T cells. No serologic changes were seen with the position 9 or 13 mutants, while some but not all β-dependent antibodies lost reactivity with the 65–67 mutant. The T-cell reactivity results were complex, with six distinct patterns noted. At least two hybridomas distinguished the position 9 vs position 13 mutants, despite their serologic identity. These results led the authors to conclude that multiple distinct regions of class-II molecules are involved in the recognition events of different T cells, and, as was seen in the case of exon and hemi-exon shuffled genes, conformational effects probably are involved in the generation of these multiple sites.

Ronchese and coworkers (120) have utilized site-directed mutagenesis to evaluate the role of the individual amino acid changes involved in the natural bm12 mutation. McIntyre & Seidman (121) demonstrated that this mutation involves three amino acid changes (Ile → Phe, Arg → Gln, Thr → Lys at positions 67, 70, and 71, respectively) in the A^b_β chain. Each of these positions of wild-type A^b_β was mutated independently or in pairs to generate all permutations between A^b_β and A^{bm12}_β. These Aβ genes were cotransfected with A^b_α into L cells, and suitably expressing clones were isolated. Staining with three monoclonal antibodies that discriminate between the parent and mutant molecule showed that the Arg → Gln substitution at position 70

was of critical importance for this serologic distinction, and the residues at 67 and 71 had little or no effect on binding. A very different picture emerged when T-cell hybridomas restricted to either I-A^b or I-A^{bm12} were tested. Only one of five such T cells showed reactivity approaching the level of the intact b or bm12 when any of the three positions was changed. These data indicate that all three residues contribute strongly to the structural features of I-A involved in antigen presentation to T cells. Thus, not only do changes in widely separated regions of the linear sequence of Aβ have effects on Ia function, but multiple residues in a small area can all play major roles in T cell–recognition events.

Results consistent with the above have been obtained by analyzing the Aβ genes of immunoselected mutant-B-lymphoma cells. Glimcher et al (122) derived a series of such mutants by treatment of the TA3 (H-2^a × H-2^d) cell with ethanemethylsulfonate, followed by negative selection with one monoclonal anti-I-A antibody plus C, then positive selection with a second monoclonal anti-I-A. Brown et al (123) have cloned and functionally characterized the A^k_β gene from one such cell created using an anti-A^k_β monoclonal for negative selection. For the A19 mutant cell line, there was a single nucleotide change in the relevant β_1 domain, changing $Gly^{67} \rightarrow Lys$. Transfection of an exon-shuffled gene containing this mutant $A_{\beta 1}$ domain and the remainder of A^d_β showed that this change accounted for the altered reactivity with antibodies and for the limited changes previously noted for A19 APC function. The fact that a mutation in this region had no discernible effect in activating some I-A^k restricted cells, while leading to a complete failure to stimulate others, again indicates either multiple regions on I-A seen differently by distinct T cells or sufficiently great differences in avidity for a single site that the response of one cell becomes unmeasurable, while the other still responds. Careful antigen dose–response analysis using transfectants bearing equal amounts of wild-type or mutant I-A will help distinguish between these possibilities.

Other groups have also begun studying transfectants created using genes bearing site-directed mutations. B. Malissen has analyzed linker-insertion mutants of the E^k_β gene and found that a single antibody reactivity can be affected by changes at either position 24 or 93 (manuscript in preparation). Some T cells respond normally to mutants at either position, while others fail to respond to changes at position 24. D. Mathis and C. Benoist (personal communication) have created mutants of the A^k_α molecule and find that a change in amino acid 75 to the A^b_α allelic residue causes loss of reactivity with both 39C and 39J antibodies, even though these monoclonals show distinct cross-reactivities on a panel of allogeneic haplotypes (124). Again, these results point to the crucial role of chain pairing and

folding (i.e. three-dimensional configuration, not linear sequence) in creating B and T cell–recognition sites on class-II molecules.

CONCLUDING REMARKS

DMGT has proved to be an extraordinarily valuable methodology for the analysis of expression and function of class-II MHC genes. Specific segments of the noncoding DNA surrounding these genes have been identified as playing critical roles in tissue-specific expression and the response to lymphokines such as γ-IFN. The importance of cellular environment, in addition to DNA-sequence and mediator-receptor activity, has been documented for class-II-gene transcriptional activity. Some previously held views concerning Ia expression, such as the requirement for $\alpha:\beta$ chain pairing prior to membrane expression, have been confirmed, while others have been challenged, particularly those regarding expression of haplotype- or isotype-mismatched $\alpha:\beta$ pairs. The possibility that invariant chain is not required for transport of Ia to the plasma membrane has been raised by several studies.

Serologic analyses of the expressed products of transfected MHC genes have provided a clearer definition of the regions of class-II molecules controlling the epitopes seen by antibodies. Perhaps unexpected was the apparently rare involvement of polymorphic sites on more than one domain in the creation of such epitopes. At the same time, direct evidence for important structural interactions between domains was provided by analyses of class II–class I chimeric molecules or unusual recombinant A_β-A_α pairs. The use of nonhematopoietic recipient cells for class-II gene transfer resulted in additional insights into the physiology of APC function and the role of nonclonotypic membrane molecules such as LFA-1 or L3T4 in T cell–APC interaction.

Attempts to define the regions of class-II molecules involved in allorecognition or MHC-restricted antigen recognition by T cells have been both rewarding and problematic. The expected special role of the highly polymorphic α_1 and β_1 class-II domains in T-cell recognition has been amply documented by this approach. At the same time, efforts to define particular subregions or residues within these domains as "the" critical one(s) involved in the recognition process have led to conclusions anticipated by earlier studies on spontaneous K^b mutants and by antibody blocking. It is now clear that the α_1 and β_1 portions of class-II molecules (as for the α_1 and α_2 domains of class-I molecules) interact to produce multiple complex combinational or conformational sites with distinct involvement in responses of individual T lymphocytes. While some of this complexity may be more apparent than real and may disappear as more data are

gathered, such findings nonetheless imply that other techniques will be required for determining how T-cell receptors, antigen, and MHC molecules physically perform their roles in the recognition process. Furthermore, the results of analyzing one model system in this regard may not give an answer entirely generalizable to another.

One satisfying outcome of the work to date on MHC structure-function relationships is the close concordance between studies on class-I and class-II genes. It has been repeatedly noted (see 2, 3) that in their membrane forms both molecules consist of four extracellular domains, three of which have internal S-S bonds and two of which, membrane-proximal domains, show significant homology to immunoglobulin constant regions. The data from exon-shuffling studies and $\alpha:\beta$ pairing studies support the view that class I α_1 and α_2 and class II α_1 and β_1 domains are functionally equivalent, interacting in each case to form a quaternary structure seen by the T-cell receptor in a highly specific manner. This view implies that recognition by class I–restricted vs class II–restricted T cells is fundamentally the same process, differing only in fine specificity and consistent with the emerging data on shared V_β and V_α receptor gene usage by T cells of both restriction types (125).

With little doubt the next few years will see a rapid accumulation of data refining the mapping studies reviewed here on regulatory or functional regions of class-II MHC genes and their products. Even without new physical data on protein structure, the extensive cataloging of mutational variation vs functional effects is likely to lead to new insights into the relationships among protein sequence, molecular assembly/expression, and the events of T-cell receptor and/or antigen binding to MHC products. An increasing number of new (transgenic) mouse strains possessing unique MHC phenotypes will become available for dissecting the role of the MHC-gene products in determining the expressed T-cell repertoire and Ir phenotype. The excitement generated by the initial application of DMGT to the analyses of MHC genes has continued unabated, and the prospects for substantive progress in our understanding of these immunologically critical genes and molecules through application of this approach are excellent.

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CONTENTS

Ann. Rev. Immunol. 1986. 4 : 317–38

THE REGULATION AND EXPRESSION OF *c-myc* IN NORMAL AND MALIGNANT CELLS[1]

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INTRODUCTION

Lymphocytes exist in a dynamic state that alternates between small quiescent cells and large, antigen-activated, proliferating cells. Their failure to activate properly or to turn off a proliferative program is a likely step in the development of lymphoid malignancies. Understanding the genetic mechanisms that regulate the expression of the alternately quiescent and activated phenotypes is a fundamental question for immunology and oncology. Significant progress in the identification of DNA elements that appear to play a role in the regulation of proliferation and differentiation has come about as a consequence of the isolation of oncogenes, pieces of genetic information that are involved in the malignant transformation of cells.

Oncogenes were first isolated as part of the small genomes of rare RNA tumor viruses, called *acute transforming retroviruses*. These viruses efficiently transform cells in culture and in animals. Acute retroviruses are

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distinct from slow transforming retroviruses that, by contrast, do not possess oncogenes. Accordingly, slow viruses are common in nature, do not transform cells in culture, and only rarely cause tumors in vivo.

Normal vertebrate cells contain similar, but nonidentical, copies of the oncogenes carried by the acute retroviruses. These cellular genes, therefore, have been termed *cellular oncogenes* (*c-oncs*) or *proto-oncogenes*. It has become clear that viral oncogenes arose as a consequence of genetic recombination between slow retroviruses and cellular oncogenes (1). Unlike viral oncogenes, however, proto-oncogenes do not transform cells. On the contrary, proto-oncogenes are widely expressed in normal cells and are thought to be fundamental to cellular metabolism. However, a role for oncogenes in spontaneous transformation has been strongly suggested by the finding that tumor cells often possess proto-oncogenes that are genetically altered, compared to a normal cellular counterpart from the same individual.

In this review, we focus on the role of one particular oncogene, *c-myc*, in both normal and neoplastic growth. In almost all differentiated cell types, *c-myc* is ubiquitously expressed. Structural alterations in the *c-myc* gene and abnormal expression associated with them have been observed in numerous tumors, most often B- and T-cell malignancies. In addition, *c-myc* expression is differentially regulated in resting versus activated cells; lymphocytes are one such model system. The regulation of *c-myc* gene expression is discussed as it relates to lymphoid and other cell types.

Data obtained from three experimental strategies that provide clues concerning the role of *c-myc* in normal and neoplastic proliferation are reviewed. These strategies include the following models: (*a*) naturally arising cancers that show associated alterations in *c-myc*, (*b*) normal and malignant cells whose growth state can be experimentally manipulated and correlated with *c-myc* expression, and (*c*) cultured cells and transgenomic mice harboring exogenously introduced *myc* sequences. Such model systems support a picture in which *c-myc* gene expression is highly regulated and intimately involved in the proliferative response of a number of cell types and can, when abnormally expressed, play a causative role in malignant transformation.

Structure of v- *and* c-myc

MC29 is the prototype avian defective retrovirus that carries the transforming *v-myc* sequence (2). Three additional replication-defective, oncogenic avian retroviruses (CMII, OK10, and MH2) have been classified with MC29 on the basis that they also possess common *myc* sequences (2). The transforming gene of MC29 has been shown to be a genetic hybrid composed of a partial retroviral structural gene, delta *gag*, linked to the *v-*

myc sequence (3). Delta *gag-myc* gene proteins are thought to be encoded by CMII and OK10 (4). In contrast to the single oncogenes carried by MC29, CMII, and OK10, the MH2 virus encodes two cellular-derived sequences, delta *gag-mil* and *myc* (5, 6). The *v-myc* gene carried by the MC29 family of viruses is composed predominantly of sequences derived from the last two chicken *c-myc* exons.

The mature message for the *c-myc* oncogene is composed of three exons in man (7), mouse (8), and chicken (9), only the last two of which are translated into protein. The first exon is large (greater than 400 basepairs in length) and untranslated, and therefore, has been suspected to play some role in the regulation of *c-myc* expression, most probably at a posttranscriptional step. However, no supporting evidence for a clearly defined function of the first exon has come to light as yet. The *c-myc* gene is transcribed from two distinct promoters, located close to each other (7, 8). Though the relative rate at which these promoters are used in different cells is somewhat variable, no true selective regulation of the two promoters has been documented, nor has there been any evidence of a qualitative difference in the two transcripts, with one possible exception (10).

The protein encoded by *c-myc* travels at an anomalous molecular weight in SDS polyacrylamide gels (11, 12). This property appears to be inherent in the primary amino acid structure, as an *E. coli*–produced myc protein, encoded by a cloned cDNA, does the same (13). Thus, no extensive posttranslational modifications have to be invoked to explain the apparent size of the protein, although it is known that the protein is phosphorylated in its natural environment (11). Also, the *myc* protein travels as two distinct bands on SDS gels (11, 12), but the significance of this, if any, is not known. Both the message and the protein specified by the *myc* gene have a relatively short half-life (10, 11). Such rapid turnover rates are well suited for a protein whose expression needs to be regulated quickly, so that changes in transcription rates can be immediately translated into changes in protein amounts.

The putative regulatory regions of the *myc* gene have been identified by DNAase-I hypersensitivity (14, 15). DNAase-I-hypersensitive sites mark perturbations in the regular chromatin structure that are usually (and possibly always) caused by the binding of nonhistone factors to DNA. Thus, the five consistently observed hypersensitive sites of human *c-myc* lie at or near sequences that are likely to be functionally relevant. Each promoter is associated with a hypersensitive site just upstream of the TATA motif. Another site located almost 2 kilobasepairs (kb) upstream is found within a region of strong sequence conservation between mouse and man, suggesting functional significance. The two remaining sites are located near sequences now recognized as close to the consensus sequence for the

binding of nuclear factor 1, an abundant cellular protein with no obvious tissue specificity (16, 17). At least one of the two sites was indeed shown to bind this protein in vitro (14). The function of nuclear factor 1 is currently unknown, although it is important for replication in the context of the adenovirus, where such a binding site has also been identified (18). The putative regulatory region of *c-myc*, as defined by these hypersensitive sites, extends about 2 kb upstream of the transcriptional start sites (14).

ALTERATIONS IN *c-myc* OBSERVED IN TUMORS

A role for *c-myc* in spontaneous transformation has been strongly suggested by the finding that tumor cells often possess a *c-myc* gene that appears somatically altered when compared to a normal cellular counterpart. Such structural alterations near or in the *c-myc* gene include chromosomal translocations, retroviral insertions, and amplification. Here we review each category of *c-myc* gene modification and associated changes in expression.

Retroviral Insertion

Slow transforming retroviruses do not possess oncogenes, yet they are able, albeit infrequently, to cause tumors in vivo (1). Analysis of those rare tumors caused by slow retroviruses led to the identification of preferred integration sites associated with specific tumors. In this way, the first structural alteration in the *c-myc* locus was demonstrated as an integration of avian leukosis virus (ALV) adjacent to *c-myc* in a majority of ALV-induced bursal lymphomas (19, 20). Tumor-bearing birds show transformation events that are monoclonal in origin with respect to ALV integrations. ALV integration occurs predominantly 5′ of the second *c-myc* exon and in the same transcriptional orientation as *c-myc*. ALV insertion induces transcriptional activation of the *c-myc* gene resulting in hybrid transcripts that initiate in the ALV long terminal repeat (LTR) and continue through into the *c-myc* gene (19, 20). Such a mechanism of activation has been termed *promotor insertion.* In tumors where the alignment of the provirus and *c-myc* precludes a hybrid transcript (20), the stimulation of *c-myc* transcription has been ascribed to an LTR enhancer, an element that can, independent of orientation, increase utilization of a nearby promotor.

Recently it has been shown that retroviral insertions appear to be involved in the genesis of murine T lymphomas. Of the T lymphomas arising in AKR mice, a strain prone to leukemia, 15–20% were found to have MCF-like retroviral insertions near *c-myc* (21, 22). Most insertions lie upstream from, and in opposing transcriptional orientation to, *c-myc*, although insertions 3′ to *c-myc* have also been observed (22). Elevated levels

of normally initiated *c-myc* mRNA, relative to thymocyte *c-myc* mRNA levels, have been observed in such tumors (22). The orientation of the LTR promotor relative to *c-myc* and the structure of the mRNA exclude a promotor insertion model. Therefore, it has been suggested that *c-myc* activation is probably mediated by the LTR enhancer. In addition, the disruption of putative 5′ regulatory sequences in the *c-myc* gene may be functionally important in some insertions.

In mouse strains other than AKR and in rats, proviruses near *c-myc* also have been found in several T lymphomas induced by murine leukemia viruses (23, 24). Additional sites of frequent integration, distinct from *myc*, have been described in rat thymomas (25, 26). One such integration region has a murine homologue on *myc*-bearing chromosome 15, which is, however, at a long distance from *c-myc* (27). Furthermore, over 50% of early T-cell lymphomas resulting from MuLV infection of newborn mice show integrations at a locus designated *pim-1* (28), which is on an altogether separate chromosome from that of *c-myc*. Thus, slow leukemia viruses seem able to induce lymphomagenesis by several routes, integration near *c-myc* being one.

In summary, the repeated isolation of avian B-cell and murine T-cell tumors that show slow retroviral insertions adjacent to the *c-myc* gene strongly implicates alterations of *c-myc* expression in the genesis of these tumors. The exact site and orientation of integration can vary. In both B- and T-cell tumors, *c-myc* is overexpressed relative to normal cell controls, although it is difficult to make comparisons because the exact nature of the tumor precursor cells is unclear. Also, one must consider the possibility that retroviral insertion into or near regulatory sequences disrupts the normal control of *c-myc* expression, a situation that may be more important than simple overall increase in steady-state mRNA levels.

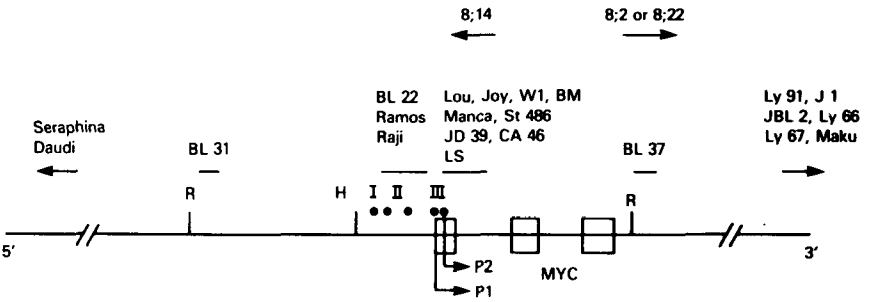
Translocations

Murine plasmacytomas and human Burkitt's lymphomas (BL) are characterized by consistent reciprocal chromosomal translocations involving the *c-myc* locus and one of the three immunoglobulin (Ig) loci (29). Thus, following the translocation event, *c-myc* is juxtaposed with either the kappa, lambda, or heavy-chain genes (for review see 30–32). In both types of tumors, the joining of the *c-myc*-containing chromosome with the heavy chain–containing chromosome is most frequent, occurring in more than 80% of tumor isolates. The universality of one of the three types of translocations in BL and plasmacytomas is one of the most compelling reasons to associate this tumor causally with the translocation event.

Burkitt's lymphoma (BL) is a B-cell neoplasia that has its highest incidence in central Africa, where it occurs especially among children and is

usually associated with Epstein-Barr virus (EBV) (33). However, in other areas of the world, a consistent association between BL and EBV is not observed (34). Thus, although the presence of EBV may contribute to the overall occurrence of BL, possibly by acting as a B-cell mitogen, this virus appears not to be necessary for tumorigenesis. Although the expression of immunoglobulin genes places Burkitt's lymphoma cells in the B-cell lineage, the exact nature of the precursor cell giving rise to this tumor is unknown.

What characterizes the chromosomal translocation events that occur in Burkitt's lymphomas? In most cases, the *c-myc* gene located on chromosome 8 band q24 is joined by means of a reciprocal translocation event to the mu heavy chain, located on chromosome 14 band q32. Then *c-myc* is found on chromosome 14 q^+, and the two gene loci are connected in a transcriptionally opposing fashion (31–33). As a consequence, *c-myc* is not transcribed from promoters belonging to the mu heavy chain but is transcribed from the normal *c-myc* promoters or crytic promoters within the *c-myc* locus. The actual chromosomal breakpoints within the mu locus and near the *myc* locus vary, but usually they occur within the mu switch sequences and near the beginning of the *c-myc* gene (see Figure 1). The precise role of the juxtaposed Ig sequences is unknown. The well-characterized transcriptional regulatory sequences within the heavy chain



Regions of the human c-myc locus containing the crossover points to immunoglobulin gene loci

Figure 1 Chromosomal breakpoints relative to c-myc for various Burkitt's lymphomas. The bars denote the approximate areas in which c-myc is joined to the immunoglobulin loci. The breakpoints occurring very far 5′ and 3′ have not been located as yet, as indicated by the broken lines. Breakpoints 5′ of c-myc are for the t(8;14) translocations, and breakpoints occurring 3′ are for the t(2;8) and t(8;22) translocations. The boxed regions represent the 3 c-myc exons. The hypersensitive sites I, $II_{1,2}$ and $III_{1,2}$, shown as dots, mark the putative control region of c-myc. P1 and P2 are the two c-myc transcription start sites. The translocations are described in the following references: 7, 8, 14, 35–43. R = Eco R1, H = Hind 3 restriction enzyme sites.

locus, the Ig promotor and enhancer, usually do not reside on the same chromosome as the *c-myc* structural sequences.

Murine plasmacytomas are tumors of Ig-producing plasma cells. By analogy with BL, such tumors virtually always display reciprocal translocations between the *c-myc*-resident chromosome 15 band and most often the heavy-chain-resident chromosome 12 band (30, 31, 44, 45). Molecular structural analyses have revealed that such translocations generally are very similar to those described above for t(8;14) BL translocations. Translocations involving mu, gamma, and alpha heavy-chain genes have been described (46–48).

The less-well-characterized "variant" translocations found in about 15–20% of plasmacytomas and BL also involve the *c-myc*-bearing chromosome band. However, exchanges occur with a chromosome bearing an Ig-light-chain locus—in mice, chromosome 6 bearing the K locus (44), and in man, chromosome 2 (2p12) and 22 (2q11) bearing the kappa and lambda loci (32), respectively. In the BL variant translocations, *c-myc* remains on chromosome 8; this is shown by somatic cell hybrid analyses (49), in situ hybridization (37), and fluorescent chromosome sorting (35). Consequently it can be deduced, and in some cases shown by molecular cloning, that chromosome 8 scission occurs 3′ instead of 5′ to *c-myc* (35–37, 49). In general, the chromosome 8 breakpoint is at an unknown (but greater than 20-kb) distance from *c-myc*. However, in one unusual case of a BL t(8;22) translocation, the lambda light chain was shown to be within two kb of *c-myc* (36).

In t(2;8) BL, it has been shown by somatic cell hybrid and in situ chromosome analyses that the kappa constant region resides on the 8 q^+ chromosome that also bears *c-myc* (38, 49). In one BL t(8;2) translocation, the breakpoint on chromosome 2 is just 5′ to the kappa joining region (35). Thus, unlike the t(8;14) translocations, the *c-myc* locus and a transcriptional regulatory enhancer (kappa) are contained on the same chromosome, albeit at a great distance apart.

In plasmacytomas bearing t(6;15) translocations, the breakpoint on chromosome 6 is just 3′ to the kappa joining region, but 5′ of the kappa enhancer and constant region (50). As with variant BL, such plasmacytomas demonstrate chromosomal breakpoints at a long distance from *c-myc*. A variety of such breakpoints occurs within a small region of chromosome 15 that has been termed *pvt-1* (50). Interestingly, thymomas bearing slow retroviral insertions in this same chromosomal region have been described (51). Thus, *pvt-1* may play a regulatory role for *c-myc* through long-range structural changes in chromatin or via a hypothesized protein product (51).

In summary, the less common "variant" translocations are structurally

quite distinct from the translocations involving the Ig heavy chain. In most cases the scission on the *c-myc*-bearing chromosome is at an indeterminate, long distance from *c-myc*. Additionally, in BL it is clear that the breakpoint on chromosome 8 occurs 3′, not 5′, to the *c-myc* gene. It is important, however, that variant BL and plasmacytomas show levels of *c-myc* mRNA that are roughly equivalent to those in BL and plasmacytomas bearing heavy-chain translocations.

EFFECTS OF TRANSLOCATIONS ON *c-myc* EXPRESSION The large number of translocation points that have been analyzed leads to a situation that is both confusing and educational. The variety of breakpoints adjacent to *c-myc* (see Figure 1) makes simple explanations regarding their effects difficult. On the other hand, with enough accumulated data, the crucial change(s) may finally be known. Two important questions must be considered in analyzing the consequences of these chromosomal translocations. First, how are the levels of *c-myc* mRNA altered in BL cells, and second, is the structure of the *c-myc*-encoded protein changed?

When compared to nondividing, normal peripheral blood lymphocytes, BL cells contain relatively high levels of *c-myc* mRNA (K. Kelly, U. Siebenlist, unpublished observation) but show the same or slightly higher levels of *c-myc* expression in comparison to proliferating, nontumorigenic B cells (38). Similarly, plasmacytomas and LPS-stimulated, dividing spleen cells display equivalent *c-myc*-mRNA levels (52). These data argue that a simple quantitative increase in *c-myc* alone does not satisfactorily explain transformed growth. The initial finding of *c-myc* translocation to Ig genes led to speculation that the *c-myc* gene would show increased expression because it was subject to the Ig regulatory mechanisms. However, it is now clear that this is not the case in the common t(8 ; 14) translocations since the immunoglobulin enhancer sequences, thought to be responsible for the very high level of immunoglobulin mRNA expression in B cells (53, 54), are not usually rearranged into the *c-myc* locus (32). Currently uncharacterized transcriptional regulatory sequences may be located 3′ of heavy-chain switch regions.

Do the translocations cause mutations within the *c-myc* coding sequence that create an altered protein in a manner analogous to the *c-ras* mutations? Although translocated *c-myc* genes can clearly be mutated, no uniform, consistent nucleotide changes have been observed. Furthermore, several translocated *c-myc* genes are known to conserve their protein coding sequences (7, 55, 56). Thus, the tumorigenic phenotype of these BL cells cannot usually result from a modified activity of the *c-myc* protein itself. On the other hand, many mutations have been observed in the

nontranslated first exon of *c-myc* (36, 38, 57). In addition, many translocations interrupt or eliminate the first exon from the main body of the *c-myc* gene. Two points must be considered, though. First, just as in the coding sequences, no consistent mutations in the first exon have been found, i.e. neither the extent of these DNA-base changes nor their positions conform to any pattern. Second, several of the Burkitt's lymphoma lines do not appear to have any gross alterations in the first exon as determined by S1 nuclease analyses (37, K. Kelly, unpublished observation). These data suggest that alterations in the *c-myc* protein sequence or the first exon are not required, but the data do not address the possibility that amino acid changes in *c-myc* or changes in the first exon may lead to an increased tumorigenic potential.

We are left then to consider the deregulation of *c-myc* transcription in BL and plasmacytomas as the crucial consequence of translocations, and for this there is good evidence. Although there is no dramatic overall increase in *c-myc* transcription, all or almost all of *c-myc* mRNA originates from the translocated allele, with little or no transcription originating from the germline allele (8, 32, 38, 57). Analyses of BL and plasmacytoma cells often show somatic alterations within the transcriptional unit of the translocated *c-myc* gene that allow its distinction from the normal allele's transcript. In addition, fusion of BL cells to mouse plasmacytoma cells results in cell lines carrying a chromosome harboring either the translocated or germline human *c-myc* gene. In such hybrid cells, only the translocated *c-myc* gene is expressed (58, 59). These observations have led to the suggestion that *c-myc* is negatively regulated by a mechanism that can repress the normal gene but not the translocated allele. Repression may occur via a negative feedback regulator sensitive to high levels of *c-myc* protein in the cell (32), but no direct evidence has been documented.

The data discussed so far support the notion that the regulation of *c-myc* is fundamentally upset in BL and plasmacytomas and that this deregulation may be due to an effect of the translocations on regulatory elements 5′ of the gene (see Figure 1). Putative regulatory sites upstream from the *c-myc* gene defined by DNAase I hypersensitivity are interrupted or eliminated in almost all BL t(8;14) and plasmacytoma t(12;15) translocations. In the variant translocations and in those t(8;14) translocations involving breakpoints 5′ of the regulatory region of *c-myc*, one must hypothesize long-range *cis*-acting effects of the translocation upon regulatory sequences as a consequence of, for example, changes in chromatin structure. If the disruption of these regulatory sites is essential, then the rare translocations occurring further 5′, leaving the putative control elements intact, might also have an effect on these sites. Indeed, the hypersensitive sites are altered by a

translocation in the BL31 cell line that occurs far 5′ to *c-myc* (see Figure 1) (14). The nontranscribed germline allele presents a chromatin pattern different from that of the translocated allele. Thus, the translocation event appears to have an effect at a distance, deregulating *c-myc* expression. The normal germline *c-myc* gene in BL31 exhibits a particularly strong, singular hypersensitive site, a possible site of action for a negative regulatory element, since this allele is silent.

All analyzed translocations in which the putative control region has been left connected to the body of the *c-myc* gene feature the presence of an immunoglobulin enhancer sequence on the chromosome that contains *c-myc*. As we indicated earlier, this is also true for the variant, t(2;8) translocations in which the regulatory region remains intact as the breakpoints occur 3′ of the *c-myc* gene. Perhaps the immunoglobulin enhancer exerts a direct or indirect effect at a distance to regulate *c-myc* expression, counteracting a negative regulatory element. It may do so by directly stimulating the *c-myc* promoters or indirectly by interfering with repression. In any case, the loss or mutation of this regulatory element or the action of an immunoglobulin enhancer at a distance may prevent a proper downregulation of the *c-myc* promoter activity, thus creating a prerequisite for tumorigenesis. In conclusion, we are left with the picture of a gene apparently highly regulated, but which is deregulated in Burkitt's lymphoma.

Amplification

Unlike translocations and retroviral insertions involving the *c-myc* gene that have been demonstrated only in lymphoid malignancies, gene amplification of *c-myc* has been described in relation to a variety of cell types. Ten- to fifty-fold DNA amplifications of *c-myc* resulting in enhanced expression have been shown for two human cell lines, the promyelocytic leukemia HL60 (60) and the colon carcinoma COLO 320 (61), and in two murine osteosarcoma-derived SEWA cell lines (62). Amplification of *c-myc* is not commonly associated with tumor tissue of the above classifications isolated from different individuals. In fact, the degree of *c-myc* amplification varies depending upon whether SEWA cells are selected in vivo, where greater amplification is observed, or grown in vitro, where amplification is decreased (62). Thus, *c-myc* amplification may be a secondary selection for increased tumorigenicity, as opposed to a primary genetic lesion leading to malignancy. In addition, a causal role for *c-myc* amplification in contributing to degree of malignancy has been suggested by the observation that five out of five highly malignant small cell lung carcinoma (SCLC) variants show *c-myc* gene amplification, while SCLC of the less malignant nonvariant type do not necessarily display such amplification (63).

THE REGULATION OF *c-myc* EXPRESSION RELATIVE TO GROWTH IN NORMAL AND NEOPLASTIC CELLS

Understanding the normal regulation of a gene allows insight into the potential function of a gene product as it relates to the changing phenotype of a cell. An obvious question concerns the expression of *c-myc* as it relates to the proliferative capacity and/or differentiated phenotype of a cell. Systems exist that allow the in vitro manipulation of cell growth, both in driving a quiescent cell to proliferate and in inducing the differentiation, accompanied by inhibition of proliferation, of other cell types. Such experimental models demonstrate that increased *c-myc* expression is closely associated with the entry of a cell from G0 into the cell cycle, while almost complete shutoff of *c-myc* expression accompanies the inhibition of proliferation associated with differentiation.

The Definition of Signals Required for Cell-Cycle Progression

To investigate the regulation of *c-myc* expression relative to growth, researchers have used in vitro systems that allow the manipulation of cell-cycle progression by defined, growth-inducing agents. These systems have included mitogen-activated lymphocytes and immortalized fibroblasts, whose growth factor requirements in vitro have been well described. In vitro studies utilizing B and T lymphocytes have defined at least two sequentially dependent positive signals required for progression from the quiescent, G0 state to the DNA-synthetic, S phase. In these cells, the first signal is most often stimulated in vitro by polyclonal mitogens and may be comparable with antigen binding to its matching receptor. In T cells, a subsequent, second signal is provided by the T cell–derived growth factor, interleukin 2 (IL-2) (64). By analogy, a second signal appears to occur (65) in B cells mediated by one or the other of at least two distinct, lymphocyte-derived B-cell growth factors (BCGF-I and -II).

Similarly, immortalized fibroblasts, e.g. 3T3 cells, also require two temporally separated signals in order to transit through G1. The growth factor PDGF has been shown to act on quiescent cells in the initial phase of G1, while EGF and somatomedins are required for the progression of PDGF-primed cells through a later stage of G1 (66).

Cell-Specific Induction of c-myc *by Agents That Initiate a Mitogenic Response*

The role of *c-myc* expression has been investigated in the mitogenic response of normal lymphocytes. Almost undetectable in resting lympho-

cytes, *c-myc* mRNA is induced approximately 20-fold between 1 and 2 hr after the addition of Con A to lymphocyte cultures. This increase in *c-myc* expression precedes by several hours increases in general RNA synthesis and replicative DNA synthesis (67). Qualitatively and quantitatively similar results concerning *c-myc* induction have been found following the stimulation of spleen cells by the B cell–specific mitogen, LPS (67).

The observation that *c-myc* mRNA levels dramatically increase promptly after the delivery of a proliferation signal to resting lymphocytes suggests that *c-myc* expression may be an important component in controlling the transit of cells through the cell cycle. The generality of such a control mechanism is supported by the fact that quiescent BALB/c and NIH-3T3 cells also show a rapid, approximately 40-fold induction of *c-myc* mRNA subsequent to treatment with PDGF (67) or other growth-promoting agents (such as serum or TPA) that mimic PDGF activation (67, 68). The PDGF-stimulated induction of *c-myc* in fibroblasts has been shown to result at least in part from transcriptional activation rather than from changes in mRNA stability (69).

The Regulation of c-myc *by Late-Acting Growth Factors*

The action of late-acting growth factors such as EGF and insulin on *c-myc* induction differs between immortalized fibroblast lines. EGF alone will stimulate a proliferative response in NIH-3T3 cells but not in BALB/c-3T3 cells (67, 70). It has been observed that EGF and insulin do not induce *c-myc* mRNA in quiescent BALB/c-3T3 cells (67), but EGF does promptly increase *c-myc* mRNA levels in NIH-3T3 cells (70). Therefore, it appears that the late-acting growth factor, EGF, can mediate *c-myc* induction when it acts upon a 3T3 cell that proliferatively responds to EGF binding. By analogy, noncycling human T cells that have been primed to express IL-2 receptors (71) show induction of *c-myc* mRNA and a proliferative response following treatment with recombinant IL-2 (K. Kelly, unpublished observation).

In summary, *c-myc* mRNA is induced by agents that initiate the first phase of a proliferative response in lymphocytes (Con A and LPS) and fibroblasts (PDGF). Further, *c-myc* mRNA levels are modulated in a primed cell by growth factors such as EGF and IL-2 that act subsequently. A strong relationship exists between the level of *c-myc* mRNA expression and the proliferative capacity of a cell population. In vivo correlates also have been reported. Regenerating rat liver cells display rapid induction of *c-myc* mRNA following partial hepatectomy (72). Also, human placental cytotrophoblasts show developmental changes in *c-myc* mRNA expression that parallel the proliferative activity of the cells (73).

The prompt induction of *c-myc* mRNA within 2 hr suggests that the *c-*

myc gene is regulated directly by the biochemical events that immediately follow PDGF binding in fibroblasts and mitogen binding in lymphocytes. Indeed, inhibition of protein synthesis does not inhibit, but superinduces *c-myc* expression (67, 70). It seems reasonable to suggest that the *c-myc* encoded protein is an intracellular mediator of mitogen binding. However, it can be deduced from a variety of systems that increased levels of *c-myc* mRNA alone, in the absence of late-acting growth signals, are not sufficient to stimulate proliferation.

An important question concerns whether *c-myc* expression is regulated with regard to the "cycling" phases of the cell cycle, G1, S, G2, and M. Recent data indicate that actively proliferating normal and tumor cells show comparable levels of *c-myc* mRNA and protein throughout G1, S, G2, and M (74). In addition, cells blocked by nutrient deprivation in G1, unlike cells blocked in G0 by growth-factor deprivation, demonstrate minimal decreases in *c-myc* levels (74).

The Regulation of c-myc *Expression with Differentiation and Cessation of Proliferation*

Just as *c-myc* transcription is upregulated in cells induced to proliferate, it is downregulated in cells induced to differentiate terminally. Since these differentiated cells cease to proliferate, the shutoff of *c-myc* transcription may well be an integral part of the differentiation process. Also, this confirms further the suspected direct association of cellular growth with *myc* expression. The promyelocytic leukemia cell line HL60 (75–77), the mouse erythroleukemia cell line MEL (78), the mouse myeloid leukemia cell line WEHI-3B (79), the human histiocytic lymphoma cell line U937 (77), and the mouse F9 teratocarcinoma cell line (68) can be differentiated terminally in vitro by a variety of agents, and in each case, *c-myc* expression is dramatically decreased. This decrease can occur within a few hours. In MEL cells, however, *c-myc* levels rise again thereafter, before they fall off permanently (78). This biphasic regulation may be present during the differentiation of the other cells as well. Possibly more than one mechanism is at work to downregulate the steady-state level of *c-myc* mRNA.

In this regard, it is interesting that the pattern of *myc* DNAase I–hypersensitive sites changes when HL60 cells are committed to terminal differentiation after 48 hr exposure to the inducing agents (U. Siebenlist, unpublished observation). A subset of hypersensitive sites disappears, most notably those next to the *c-myc* promoters, suggesting a transcriptional regulatory process. To what extent the downregulation of *c-myc* mRNA levels is a result of transcriptional rate changes alone is unclear and may differ in the various systems mentioned above. Recent reports concerning alpha or beta interferon(IFN)–induced downregulation of *c-myc* in Daudi

cells give conflicting data. From two reports, it appears that a post-transcriptional mechanism is at work (10, 80). However a separate publication demonstrates transcriptional turnoff (77).

Changes in the chromatin of *c-myc* induced during the differentiation of HL60 cells have a very interesting and noteworthy parallel in BL cells. The nontranslocated and nontranscribed allele in BL31 displays the same pattern as differentiated HL60 cells (14). Specifically, several hypersensitive sites are absent, including those near the *myc* promoters. Possibly the BL precursor cell underwent a process akin to differentiation, which involved a shutoff of *c-myc* and a cessation of proliferative activity. The translocation, however, might have interfered with this repressive mechanism and resulted in transcription from the rearranged *c-myc* allele. Seen in this light, the fact that *c-myc* is transcribed at all in the context of the BL cell may be very relevant for tumor formation.

Taken together, the data on *c-myc* regulation during differentiation suggest a very important role for *c-myc* not only in the activation of resting cells to proliferate, but also in the regulated cessation of proliferation. As discussed, deregulation of *c-myc* in BL may be interfering specifically with the latter regulation. Also, there may be several distinct molecular mechanisms involved in the complex regulation of this oncogene, which befits a gene whose expression appears so critically linked to growth.

INTRODUCTION OF EXOGENOUS *myc* GENES INTO CULTURED CELLS AND TRANSGENIC MICE

An important question concerns the functional activity of *c-myc*. Does a change in *c-myc* expression alone result in malignant transformation or might a more limited phenotypic change be defined? In order to approach these questions, DNA-mediated gene transfection has been utilized to introduce experimentally manipulated cellular and viral *myc* genes into defined cells.

Avian cells infected with *myc*-bearing viruses demonstrate morphological transformation in vitro. In addition, such cells show increased proliferative capacity defined by shorter cell-doubling times and growth in semisolid agar (81). However, infected fibroblasts are not immortalized and are not tumorigenic in nude mice (81). Similarly, there are reports that MC29-infected chicken-cell DNA (82) and cloned MC29 DNA (83) cause morphological transformation of immortalized, murine NIH-3T3 fibroblasts. In contrast, others utilizing heterologous promotor-driven *c-myc* sequences and immortalized murine fibroblasts, (84, 85) or cloned *v-myc* DNA and secondary rat embryo fibroblasts (86), observe no morphological transformation.

In view of the fact that transfected *myc* sequences often do not transform cells morphologically, the question arises as to what other phenotypic alterations are observed in such transfected cells. The induction of *c-myc* mRNA by mitogenic growth factors suggests that *c-myc* is an intracellular mediator of mitogen binding. This concept is supported by the observation that constitutive expression of an exogenously introduced *c-myc* gene in BALB/c-3T3 cells results in increased sensitivity of the transfected cells to the mitogenic activity of EGF and increases the probability of cell division in the absence of PDGF (85). However, constitutive *myc* expression in various BALB/c-3T3-transfected clones grown in PDGF-deficient media leads to DNA synthesis in, on average, about 5% of the cells over a 24-hr period, compared to less than 0.5% of nontransfected cells. On the other hand, greater than 70% of cells grown in PDGF-supplemented media will synthesize DNA during the same period. Thus, constitutive *myc* expression appears only partially to replace the requirements for mitogenic growth factors. In agreement with the above results, it has been shown that *c-myc* transfected FRF-3T3 cells become sensitized to the mitogenicity of EGF and acquire the ability to grow in semisolid agar (87).

An additional experimental strategy that has been used to define the activity of *myc* functionally is based upon the introduction of two oncogenes into normal rat embryo fibroblasts (REF) to effect morphological transformation. It has been shown that SV40 promoter-linked *c-myc* genes and mutant *ras* genes could transform secondary REF, while the *ras* gene alone could not (86). Because this cooperating *myc* activity can be replaced by the use of established fibroblasts or by so-called immortalizing genes, adenovirus E1A and polyma middle-T antigen (86, 88), an immortalization-like function has been suggested for *c-myc*. It should be noted that the tumorigenic potential of secondary REF transformed by *c-myc* and *ras* is limited, while *ras*-transformed immortalized fibroblasts (NIH-3T3 cells) are more malignant (86). In contrast to the suggested immortalization function for *c-myc*, LTR-driven *c-myc* expression has recently been shown to lead to tumorigenicity in the immortalized cell lines, NIH-3T3 and Rat-2, despite a lack of morphological transformation in these cells (84).

The results obtained from *myc*-transfection experiments into fibroblasts indicate that constitutive *myc* expression can result in a variety of phenotypic alterations. Because the various fibroblast recipient cells vary in regard to species, history in culture, and growth properties, it appears that the genetic background of the recipient cells (although all of these cells are of the same fibroblastic differentiated phenotype) sets the stage for defining a functional activity for *myc*. In summary, constitutive expression of *myc* in established Balb/c-3T3 fibroblasts alters the growth properties of these cells

as defined by a reduced requirement for the growth factors contained in serum. In addition, exogenously introduced *myc* contributes to the tumorigenic potential of NIH-3T3 and Rat-2 fibroblasts. Whether these separately defined functions of *myc* are related has yet to be clarified.

Transfection experiments are limited in the sense that only a restricted number of differentiated cell types can be examined. Moreover, alterations in cellular phenotype associated with the in vitro culture history of cells complicate interpretations. An experimental strategy that attempts to overcome these limitations uses transgenic mice. Thus, when transgenic mice possess a *c-myc* gene under the control of a glucocorticoid-responsive mouse mammary-tumor–virus (MTV) LTR promotor, the mice develop monoclonal mammary adenocarcinomas (89). Because the tumor-cell precursors most likely are capable of responding to MTV-regulatory hormones, over-expression of *myc* may be an important factor in the development of these tumors. The monoclonality of the tumors that arise suggests that high levels of *myc* expression alone are not sufficient for tumor induction, but *myc* overexpression can be a contributing factor in the tumorigenicity of mammary adenocarcinomas. Future experiments directed at targeting increased *myc* expression to other cell types in transgenic mice may help to clarify the variety of differentiated cellular backgrounds that are sensitive to *myc* deregulation.

BIOCHEMISTRY OF THE *myc* PROTEIN

The protein specified by *c-myc* has been shown to localize in the cell nucleus (11, 90, 91), in a way similar to that of *v-myc* protein encoded by the various isolates of *myc* containing avian retroviruses (92–94). The protein appears to have some structural similarity with the E1A-transforming protein of adenovirus (95), also localized in the nucleus and known to stimulate the transcription of other genes (96, 97). Therefore, *myc* may well function as a transcriptional regulator or even be involved in replication. In fact, a *c-myc* construct transfected into cells activates the transcription of an exogenously introduced heat-shock protein gene upon expression of the *myc* protein (98). Although more evidence is required to make definitive conclusions, a *trans*-activating role for *myc* would well explain the pleiotropic growth-related functions that we ascribe to this gene.

The *myc* protein may accomplish this role by direct DNA interaction in the nucleus. Both *c-myc* and *v-myc* proteins have been shown to have affinity for DNA (90, 91, 93). In addition, the *E. coli*–synthesized *myc* protein, as specified by a human cDNA clone, actually can be demonstrated to have a relatively high nonspecific binding constant for DNA in vitro (13). On the other hand, a major portion of *myc* in the cell reportedly interacts

with the nuclear matrix organization and remains there even after extensive digestion of DNA (91). Interestingly, the E1A protein appears not to bind to DNA directly, but rather stimulates transcription indirectly (99, 100). Whether the *myc* protein acts directly on DNA or indirectly through association with other proteins, and whether it does so within the framework of the nuclear matrix, is unclear at present. Nonetheless, the data suggest that *myc* is involved in nuclear regulation aimed at activating the cell to grow.

CONCLUSIONS AND PERSPECTIVES

The data discussed in this paper strongly implicate a link between *c-myc* expression and proliferative activity of a given cell. It is also clear that this important gene is highly regulated and sensitive to the environment via signals received from membrane receptors. Thus, *c-myc* may be an intracellular mediator of the extracellular environment.

Most likely, the overexpression or deregulated expression of *c-myc* observed in tumors bearing structural alterations in the *c-myc* gene, such as translocation, amplification, or adjacent retroviral insertion, contributes to tumor formation. Less clear is why genetic changes in *c-myc* are seen in some tumor types and not in others. The probability of a particular somatic change may be increased depending upon the cell type. Also, *myc* may be more tumorigenic in some cells, or others events may contribute to tumor formation in association with *myc* deregulation, and it is these other events that are tumor specific. In addition, many tumors do not appear to have undergone genetic changes of the *c-myc* locus although they do exhibit high *c-myc* expression. Thus, it is possible that genetic alterations in the pathway proximal to *myc* activation have obviated alterations in the *c-myc* structural locus.

As we discussed earlier, *c-myc* expression is correlated with cellular proliferation, and deregulated expression of *c-myc* is associated with tumor formation. However, *c-myc* expression alone is not sufficient for normal growth, nor is altered expression sufficient for tumorigenesis; much evidence suggests that at least two oncogenic events are required for transformation. Thus, *c-myc* expression is most likely a necessary, but not alone a sufficient, component of cellular growth. Whether *c-myc* expression is necessary for growth in all instances is not clear. Other proteins with *myc*-like function (possibly *N-myc* or *L-myc*) may be able to substitute for *c-myc*.

Exactly how *c-myc* mediates its effects has yet to be resolved, but a role as a pleiotropic transcriptional activator would fit current data. The work discussed in this review emphasizes the intricate network of growth regulation both of and within cells. The *c-myc* oncogene plays an important

role in these processes and presents a model system as well as entry for research into the molecular events that control growth and that can, when altered, lead to malignancy.

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CONTENTS

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REGULATION OF THE ASSEMBLY AND EXPRESSION OF VARIABLE-REGION GENES

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INTRODUCTION

During the past several years, it has become increasingly evident that the regulation of immunoglobulin (Ig) gene assembly and expression is intrinsically related to the progression of B-cell precursors to the B-cell differentiation stage. The general focus of this review is to describe in detail the mechanism of Ig variable-region gene assembly and to explore the possible regulatory mechanisms that the cell exploits to control these genomic rearrangement events. Because assembly of the variable region of the T-cell antigen-receptor genes appears to be mediated by the same molecular elements, the general principles we describe should apply to both Ig and T cell–receptor variable-region genes.

The immune system is capable of responding to an almost infinite number of antigenic challenges by producing a tremendous diversity of antibody specificities. Each antibody molecule consists of heavy (H) and light (L) immunoglobulin polypeptide chains. The carboxy terminus of H and L chains is a region of constant amino acid sequence. The constant region of the H chain is involved in a variety of effector functions, such as Fc-receptor binding and complement fixation. The amino terminus of both these chains contains a region of variable amino acid sequences (designated the variable region) that are usually unique to the chains in a given antibody molecule; the variable regions of a single H and L chain interact to form an antigen-binding pocket. It is now clear that the DNA sequences encoding

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both H- and L-chain variable regions are assembled from component gene segments (for review, see 1). The combinatorial assortment of these component gene segments, together with further diversification mechanisms that act at their junctions, provides the immune system with the capacity to encode an almost unlimited number of different H and L chains despite a limited germline genome; random pairing of the many different H and L chains further increases the diversity of antibody specificities. The fully assembled H-chain variable-region gene is located just upstream to the first constant-region gene, Cμ expressed during development; transcriptional processing mechanisms allow expression of these adjacent coding regions on the same H chain (Figure 1). A recombination mechanism independent from that used in variable-region gene assembly is capable

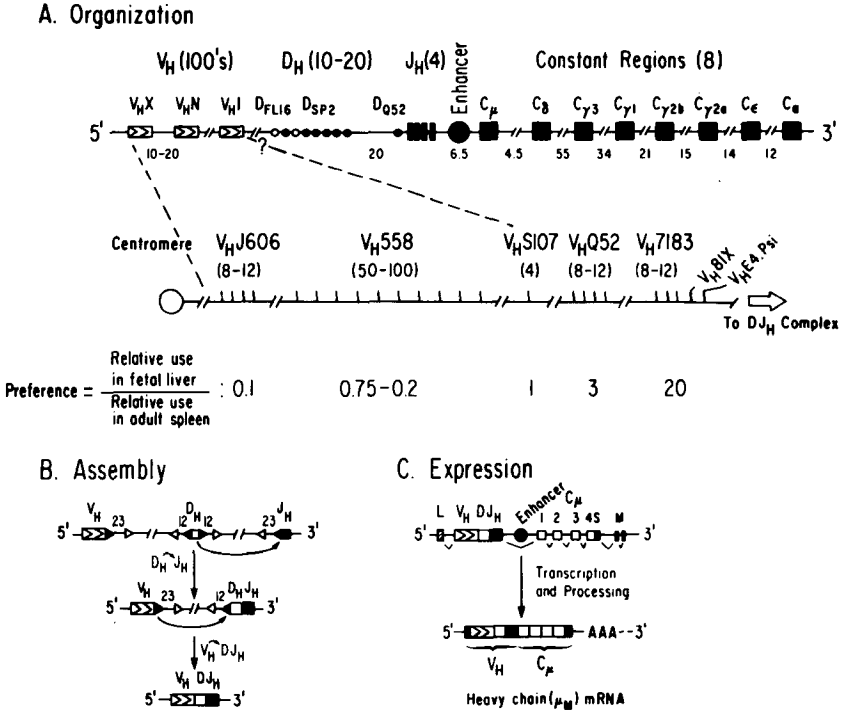

Figure 1 Organization, assembly, and expression of heavy chain locus is schematically depicted. In panel A the number of segments in each cluster is given in parentheses, with the indicated distances between segments given in kilobases. The map of the V_H locus is adapted mainly from Ref. 32 and 33, with identification of the most J_H-proximal V_H segments (VH81X and VHE4.Psi) provided by Ref. 24. The preference numbers indicating the relative use of each of the represented V_H families are taken from G. D. Yancopoulos, F. W. Alt, in preparation. The recombination recognition sequences are schematically represented in panel B; heptamers are indicated by closed triangles, nonamers by open triangles, with spacers of either 12 or 23 bp.

of replacing the Cμ gene with other constant-region genes located downstream to it while maintaining the same variable region gene; this allows a single antibody specificity to be sequentially expressed with different effector functions (2).

Ig genes are assembled and expressed by cells of the B-lymphoid lineage. The earliest identified precursor-B cells (pre-B cells) are found in the fetal liver and adult bone marrow (3, 4). These cells express cytoplasmic μ-heavy chains, but not L chains. The surface Ig-positive B cell represents the next major stage of B-cell development. It is important to note that each B cell and all of its progeny express a surface antigen receptor with a unique binding specificity. This unique specificity is maintained by the expression of just a single H- and a single L-chain variable-region gene by an individual cell. Thus, unlike most other genes, Ig genes are regulated by the principle of allelic exclusion: In a given B cell, only one of the two H-chain alleles and only one of the several L-chain alleles (see below) is expressed at the cell surface as antibody receptor (5, 6). This clonal specificity of a single B cell and its progeny is believed to be an intrinsic requirement for the proper functioning of an immune system based on antigen-stimulated clonal expansion. Only after a B cell expresses its surface receptor is it susceptible to antigen stimulation. Antigen that can bind the surface receptor triggers clonal expansion and differentiation of B cells to the terminal stage of the B-cell developmental pathway, the plasma cell. These cells secrete large amounts of antibodies bearing the initial antigen-binding specificity; allelic exclusion prevents the simultaneous production of large amounts of nonspecific antibodies by the cells involved in such a response.

MODEL SYSTEMS FOR STUDYING B-CELL DIFFERENTIATION

Analysis of B-cell differentiation has benefited from the availability of tumor cell analogues representing the various stages of the developmental pathway. B-cell leukemias and lymphomas represent the surface Ig-positive B-cell stage, while myelomas and plasmacytomas represent the mature Ig-secreting stage. Comparison of the configuration of Ig genes in such lymphoid tumor cell lines and in nonlymphoid cells revealed that Ig variable-region genes are somatically assembled; the comparison also elucidated some of the mechanisms involved in the rearrangement process (1). However, insight into the dynamics of the rearrangement process required investigation of the cell stages during which rearrangement occurred. Direct studies of fetal liver allowed analysis of Ig-gene expression in a synchronously differentiating population of pre-B cells (3, 4), whereas analysis of adult bone marrow provided information concerning a steady

state, renewable population of pre-B cells and more mature B cells (7). Fusion of fetal liver cells to a myeloma line resulted in the production of fetal liver hybridomas, which provide a model system to study the gene rearrangements of a pre-B cell within the phenotypic background of a myeloma (8–11). A remarkably accurate and dynamic representation of the Ig gene–assembly process has been provided by investigations utilizing Abelson murine leukemia virus (A-MuLV)–transformed cell lines. The most valuable aspect of these lines is their ability to proceed through immunodifferentiative events when propagated in culture.

A-MuLV-Transformed Cell Lines

A-MuLV is a replication-defective retrovirus unique in its capacity to transform immature B-lymphoid cells in culture; transformation of fetal liver or adult bone marrow cells yields permanent cell lines essentially all of which represent the pre-B or earlier stages of the B-cell pathway (12–16). Analyses of such lines have provided static models of these early stages of B-cell development. More importantly, however, many of the A-MuLV transformants undergo immunodifferentiative events when propagated in culture, including assembly of H- and/or L-chain genes and H-chain class-switching events (15, 17–25). Studies of such lines have led to a number of interesting and sometimes surprising findings concerning the dynamic processes involved in the rearrangement and expression of Ig genes; strikingly, however, most of the apparently novel aspects of Ig-gene rearrangement or expression discovered in A-MuLV transformants have served to elucidate events later demonstrated to occur in normal pre-B cells.

ORGANIZATION OF IG VARIABLE-REGION GENES

Organization of H-Chain Variable-Region Genes

The variable region of the murine-immunoglobulin H-chain gene is encoded by at least three separate germline DNA elements. Most of the variable-region gene is encoded by a variable gene segment (V_H) that can code for the 98 N-terminal amino acids. The V_H segment is usually linked to a diversity segment (D segment) that encodes the next 1–15 amino acids, and the D segment is linked to a joining segment (J_H segment) that encodes the final variable-region amino acids. The V_H, D, and J_H segments are encoded in three separate clusters (Figure 1A) on chromosome 12 of the mouse (26). The 4 J_H segments lie 7 kilobases (kb) upstream of the Cμ exons, and 10–20 D segments are located from 1 to 80 kb upstream to the J_H cluster (27). The D segments have been divided into 3 families based on nucleic acid sequence homology: The single DQ52 type D lies 1 kb upstream from J_H1; the 10 DSP2–type D segments lie from 10 to 80 kb upstream of DQ52; and

the 2 known DFL16–type D segments bracket the most 5′ member of the DSP2 family (27, 28). The possibility of a few additional D segments has not been ruled out. There are approximately 200 to more than 1000 V_H gene segments, and these lie at an as-yet-undetermined distance 5′ to the D segments. The V_H segments can be subdivided into approximately 8 distinct families based on nucleotide sequence homology (there are 4 to at least 100 segments per family, with family members sharing 70–80%, or greater, homology), and the members of a given family are grouped together on the chromosome (29–33). Adjacent members of a V_H family seem to be separated by an average spacing of 10–20 kb (29–31). The known families have been ordered along the chromosome by both deletion and recombinant inbred strain mapping (Figure 1A; 29, 32, 33).

Organization of L-Chain Genes

L-chain variable-region genes are assembled from two germline DNA elements (V_L and J_L); L-chain variable-region genes do not contain D segments (1). There are two different families of L-chain genes in mammals, designated kappa (κ) and lambda (λ). These families lie on different chromosomes (26, 34, 35). In mice, κ is the predominant light chain (90% of the serum light chain), while λ is a minor component. In other species, the ratios may be more equal or even reversed.

In mice there are 4 functional J_κ segments (a fifth segment lacks a donor splice sequence) that lie several kb upstream to the single C_κ region (Figure 2; 36, 37). There are at least 200 V_κ segments grouped into families similar to those of the V_H segments (38, 39); although the V_κ segments are generally assumed to lie 5′ to the J_κ cluster, neither their relative location nor orientation with respect to the J_κ locus has been clearly determined (see below). The murine λ locus is organized in a somewhat different fashion with only 2 V_λ genes, each of which is followed downstream by 2 J_λ-C_λ units (Figure 2; 40). The λ locus may be more complex in species where λ comprises a larger fraction of the serum light chain (40). Although the V_λ and J_λ segments have not been linked in mammals, in chickens the V_λ gene cluster begins just 1 kb 5′ to the J_λ cluster (41).

VARIABLE-REGION GENE ASSEMBLY

Recognition sequences that apparently mediate site-specific recombination flank all Ig component gene segments involved in variable-region gene assembly (for review see 1); recent investigations have demonstrated that the component gene segments involved in the assembly of the T-cell antigen-receptor variable-region gene are also flanked by recognition sequences quite similar to those of Ig-gene segments (for review see 42). A

complete recognition sequence consists of a highly conserved palindromic heptamer, which directly abuts the end of the gene segment coding sequence, and a characteristic nonamer, separated from the heptamer by a nonconserved spacer region of either 12 or 23 bp (Figure 3). The length of the spacer region apparently directs the recombinational machinery: Joining only occurs between elements that are flanked, respectively, by recognition sequences containing 12 and 23 bp spacers (43, 44).

Each D segment is flanked on both its 5′ and 3′ side by recognition sequences containing 12 bp spacers, whereas the 3′ end of all V_H segments and the 5′ end of all J_H segments are flanked by recognition sequences containing 23 bp spacers. According to the 12/23 joining rule, V_H-to-D and D-to-J_H joins are thus permitted, whereas V_H-to-J_H joins are not. However, an additional level of control appears to further restrict this joining process (for more details, see below): V_HDJ_H formation is accomplished via an ordered, two-step process in which a V_H segment is appended to a preexisting DJ_H intermediate; V_H-to-D joins, although permitted according to the 12/23 joining rule, do not normally occur, and thus, DJ_H joins

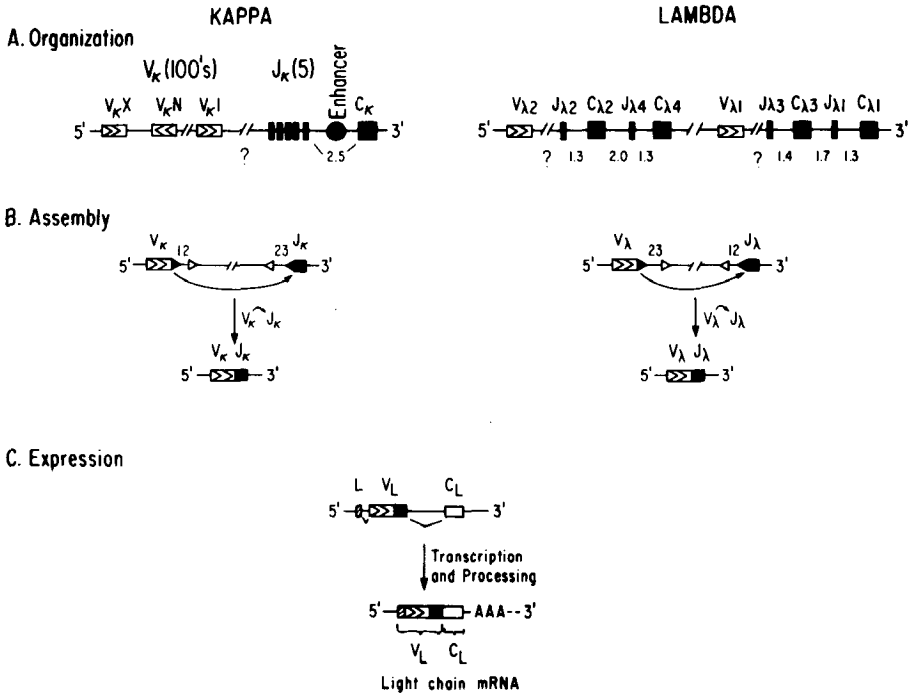

Figure 2 Light chain locus. Organization, assembly, and expression of kappa and lambda loci. When there is more than one member per cluster of segments, the approximate cluster size is given in parentheses in panel A. Distances between segments are in kilobases. The recombination recognition sequences are represented as in Fig. 1.

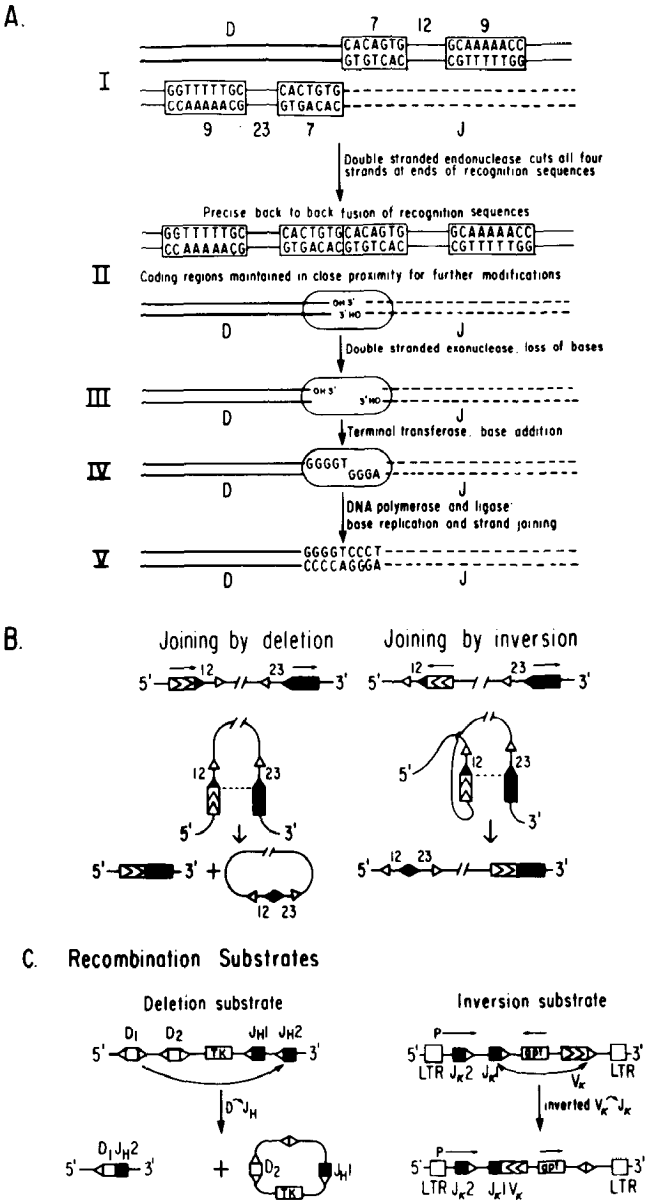

Figure 3 (A) Schematic representation of the model for imprecise joining of H-chain variable-region gene segments (99). (B) Representation of deletional and inversional modes of joining. The mode of joining seems to depend on the relative orientation of the elements to be joined. Recognition sequences are depicted as in Fig. 1. (C) Depiction of two types of recombination substrates discussed in the text. Selectable excision of the tk gene results from D-to-J_H joining as depicted on the left (109), whereas activation of an intervening gpt gene by inverted V_κ to J_κ joining is depicted on the right (100). The transcriptional orientation of the gpt gene and a promoter (P) in the viral long terminal repeat (LTR) are indicated by arrows. The recognition sequences are depicted by open triangles.

serve as the preferred intermediates in the joining process (23). Both D-to-J_H and V_H-to-DJ_H joining occur via a deletional mechanism, resulting in loss of all intervening DNA sequences (23). Thus, once formed, a preexisting DJ_H complex can still be (and is occasionally—45; M. G. Reth, S. Jackson, F. W. Alt, in preparation) replaced by joining of an upstream D to a downstream J_H. However, once assembled, a complete V_HDJ_H complex is fixed; no D segments (i.e. 12 bp spacers) remain on the chromosome to mediate any further joining events to downstream J_H segments.

L-chain variable-region genes are assembled from V_L and J_L segments. These segments are appropriately flanked by recognition sequences containing 12 or 23 bp spacers in accordance with the 12/23 joining rule (1). Because of this organization, the fully assembled V_LJ_L gene could be replaced by joining an upstream V_L to a downstream J_L; evidence exists indicating that this replacement does occur (19, 46).

Perhaps the most striking example of the 12/23 joining model came from analysis of the variable-region components of the β chain of the T-cell receptor. The J_β segments were found to be flanked by recognition elements containing 12 bp spacers and the V_β elements by recognition sequences containing 23 bp spacers; this suggested a V-to-J rearrangement mechanism reminiscent of that of Ig L chains (47, 48). However, the sequence of assembled β-chain genes suggested that D_β segments existed (47, 48). Consistent with the 12/23 joining model, these D_β segments were subsequently found to be flanked by recognition sequences containing asymmetric spacers, with the 12 bp spacer pointing towards the V_β segments and the 23 bp spacer pointing at the J_β elements (49, 50). Since direct joining of V_β to J_β and also D_β to D_β are still in accord with the spacer arrangements, it has been proposed that such rearrangements may occur and further contribute to diversification. However, the directionality of joining again may be mediated by factors in addition to the 12/23 rule that could prevent direct V_β-to-J_β joining and impose a V-to-DJ-joining order like that observed for Ig H-chain gene assembly (see below).

EXPRESSION OF INCOMPLETELY AND COMPLETELY ASSEMBLED IG GENES

Expression of Completely Assembled Variable-Region Genes

Although each germline V_H gene segment is preceded by a transcriptional promoter region that is unchanged by the rearrangement process, only rearranged V_H-gene segments are actively transcribed in mature B cells (51). The promoter of the rearranged V_H segment appears to be specifically

activated by close association (due to the rearrangement) with a tissue-specific enhancer element located in the intron which separates the fully assembled variable-region gene from the downstream constant region (Figure 1; 52–54). The H-chain enhancer is similar to other enhancer elements in its ability to enhance transcription only in *cis*, while exerting its effects in an orientation-independent manner and over large distances. In fact, it has been shown that the Ig enhancer can apparently activate an unrearranged V_H segment located 11 kb upstream to the assembled variable region, although it does not activate V_H segments further upstream (X. Wang, K. Calame, *Cell.* In press).

It has now become clear that the enhancer does not provide all the answers concerning the mechanism of high-level, tissue-specific, Ig-gene expression. The original evidence implicating an enhancer-like sequence in H-chain expression was the finding that H-chain expression was lost in the A-MuLV transformant 18–81 that had accumulated specific deletions in the intron between the J_H and Cμ region (18). However, it was also found that high-level expression could be restored by treating these enhancerless cells with lipopolysaccharide (LPS) or via fusion to a myeloma (18, 55). Later studies demonstrated that spontaneous deletions of the enhancer in hybridomas did not affect the level of Ig-gene expression (56). Such findings suggested that the enhancer may only be required for the initial activation of Ig-gene expression but is not obligatory for maintenance of transcription, or possibly, that the presence of the classical enhancer per se was not the only mechanism for high-level expression of these genes in B-cell lines.

The transcription of germ-line, unrearranged V_H gene segments in a tissue- and stage-specific manner (see below) demonstrated that sequences independent from the H-chain enhancer could be important in regulating the tissue and stage specificity of Ig-gene expression (51). Recent studies have found that sequences surrounding the V_H promoter can direct lymphocyte-specific transcription independent of the H-chain enhancer (57, 58). Transcription of completely assembled κ or λ L-chain genes appears to be under the control of similar processes (59, 60); an enhancer element has been found within the κ- (61–64) and possibly also the λ- (65) gene complex.

Expression of Incompletely Rearranged J-Associated Regions

A number of pre-B-cell lines produce truncated Cμ H-chain products that are approximately one domain shorter than normal μ chains (66). These chains were demonstrated to be produced from DJ_H rearrangements (66). Both of the major families of murine D segments contain upstream transcriptional promoters (66), as does the single member (DQ52) of the

third D family (67). Thus, the appendage of a D segment to a J_H segment leads to the generation of a primary transcript that is processed to yield a "Dμ mRNA." Each D of the SP2 and FL16 families also contains an upstream translational start codon, and if the D-to-J_H rearrangement occurs in such a way that this start codon is in the same translational reading frame as the J_H segment, the resulting mRNA is translated to yield a Dμ polypeptide chain (66). Approximately 30 to 40 amino acids are contained in the "D" portion of this protein, and each upstream D-coding sequence contains a characteristic leader sequence (66). The upstream promoter, translation start site, and leader sequences are conserved across species, suggesting that the Dμ proteins may serve some function. It is not known whether Dμ proteins get to the cell surface or are secreted; both forms of the transcripts are made (66). The DJ_β joins of the T cell–receptor β genes are also transcribed, and DNA sequence analyses indicate that these DJ_β transcripts may also encode proteins (49). No obvious function for the DJ proteins has been indicated, although evidence suggests that they could potentially play a regulatory role (see below).

Although T cells never completely rearrange their H-chain genes to the V_HDJ_H stage, they often make incomplete DJ_H joins (68). Furthermore, the H-chain enhancer appears to be active in at least a subset of T cells, allowing expression of these DJ_H joins; once again no role for such expression has been proposed (66, 69–71). Examination of T cells further reveals that unrearranged J_H regions can be actively transcribed (70). Similar analyses in B cells have not been possible because cells committed to the B lineage, but before the DJ_H rearrangement stage, have not yet been available for study. The finding that the H-chain enhancer is active without rearrangement suggests it may be activated in lymphoid cells prior to rearrangement. Similarly, germline J_κ alleles are transcriptionally active (72), and recent studies in pre-B cells that have not yet rearranged their L-chain genes demonstrate that this transcriptional activation precedes rearrangement (73). Such analyses have led to the concept that enhancers may play a role not only in gene transcription but also in targeting loci for tissue- or developmental stage–specific recombination events (51; also see below).

Expression of Germline V-Gene Segments

Expression of a single germline V_H gene was first demonstrated in a T-cell line (73a); expression level was very low and was not observed in many other T-cell lines (74, 75). Analysis of pre-B-cell lines demonstrated that a large number of germline V_H segments are capable of relatively high-level expression, with levels as high or higher than those of normal μ-chain

expression that is characteristic of the pre-B stage (51). However, such expression is limited to the very early stages of the B-cell pathway, most prominently in cells that are in the process of V_H-to-DJ_H joining (51). The stage specificity of germline V_H expression is strikingly evident in normal fetal liver, where such expression occurs at a high level just prior to the burst of full-length μ-mRNA expression (51). Germline V_H transcripts usually initiate from the normal V_H promoter, are appropriately spliced, and appear in the cytoplasm where they are probably translated into V_H chains (51). Currently, no role for the potential V_H proteins has been demonstrated, although several possibilities exist. Such a V_H protein would consist of a signal peptide followed by a single V_H domain, an organization similar to β2-microglobulin and Thy-1 glycoprotein (76–78). It is conceivable that the single Ig domains encoded by the expressed V_H-gene segments can associate with developmentally regulated cell-surface antigens in pre-B cells and be important for mediating cell-cell interactions. The V_H transcription specific to cells active in V_H to DJ_H rearrangement may reflect a targeting of these segments for rearrangement (51; also see below). Germline V_λ transcripts also have been detected, but only in cells that have rearranged both κ alleles (79).

REGULATION OF IG-GENE REARRANGEMENT AND EXPRESSION DURING THE EARLY STAGES OF B-CELL DIFFERENTIATION

The diversity of antibody specificities is due, in part, to the combinatorial assortment of the different component gene segments that can be used in variable-region gene assembly, as well as to additional sequence diversification mechanisms that are somatically imposed during or after assembly of these segments (1). All of these diversification mechanisms operate independently with respect to the two "alleles" for each species of H and L chain. Thus, for each differentiating B-lymphocyte, the potential exists for creating totally different variable-region genes at each H- and L-chain allele; assuming random assortment of the different H and L chains, a given B-cell clone could make Ig receptors with multiple specificities. However, each B lymphocyte produces a monospecific Ig receptor as a result of allelic exclusion: A given B-cell clone expresses just one of its two H-chain alleles and one of its multiple L-chain alleles (2 for κ and 4 for λ) as Ig chains that combine to form surface-Ig receptor (5, 6). Unique mechanisms appear to have evolved to ensure the generation of the monospecific B-lymphocytes that are a prerequisite to an immune system whose specificity is based upon clonal selection. The molecular mechanisms that effect allelic exclusion

involve control of V-gene assembly and must, therefore, be operative during the early, antigen-independent, stages of B-lymphocyte differentiation when these assembly events occur.

Productive and Nonproductive Rearrangements

Before we can consider the mechanism by which V-gene assembly is regulated, we must first outline another important aspect of the assembly process. The rearrangement process is by its nature imprecise (see below). In order to encode a protein, the component V and J segments must be joined in the same translational reading frame (this should occur in approximately one out of every three rearrangements), and the joins formed and the segments used must be free of in-phase nonsense codons. A rearrangement fulfilling these criteria is termed a "productive" join. Rearrangements in which the V segment is joined to the J in a different translational reading frame, or which contain a crippling mutation such as an in-phase translation termination codon, frequently are transcribed but usually encode incomplete proteins consisting of V-region fragments (80, 81). Such rearrangements are referred to as "non-productive" or "nonfunctional" rearrangements. Cell lines have been described that have V_HDJ_H rearrangements at both alleles, only one of which appears to be highly expressed at the level of steady state mRNA (18); the allele expressed at lower levels has been shown to be nonproductively rearranged (70). In such examples, it is likely that both alleles are transcribed equally, but the lower steady-state level of the mRNA expressed from the nonproductively rearranged allele results from decreased stability due to lack of ribosome protection of the untranslatable portion of the mRNA (82).

The Regulated Model of Allelic Exclusion

A simplified scheme of the antigen-independent stages of B-cell differentiation is shown in Figure 4. Our description of this portion of the pathway will focus on the regulation of Ig-gene assembly. These regulatory events are manifested in two ways: (*a*) the very strict order in which the various component gene elements are assembled and (*b*) the allelically excluded assembly of a single functional H- and L-chain variable region in each clonal lineage, which differentiates from a stem cell to yield a surface Ig-positive B lymphocyte (23, 83). H-chain variable-region gene assembly precedes L chain–gene rearrangement: A productive H chain–gene rearrangement appears to result in both the termination of further H chain–gene assembly (thus preventing production of a second H chain in the cell) and the onset of L chain–gene assembly. The various L-chain alleles are then sequentially rearranged and tested for the expression of an L chain capable of functional interaction with the preexisting heavy chain in the

cell. Production of complete Ig apparently leads to cessation of L chain–gene rearrangement, ensuring the monospecificity of the resulting surface Ig–positive B lymphocyte and its progeny. As we describe these assembly events and their apparent regulation in detail, we will refer to the experiments that have led to this general model.

REGULATION OF H-CHAIN VARIABLE-REGION GENE ASSEMBLY The ordered expression of H and L chains was suggested by analyses of fetal liver hybridomas and A-MuLV transformants that revealed both often made μ H chains in the absence of L chains; this predicted the existence of the μ-only pre-B lymphocyte (8, 14). Subsequent analysis of normal fetal liver demonstrated the existence of cell populations in vivo which produced μ mRNA and protein and further indicated that these cells arose earlier in B-cell ontogeny than cells that produced L-chain mRNA, protein, or complete Ig chains (3, 4). Examination of the Ig-gene rearrangements in pre-B-cell lines demonstrated that the μ-only phenotype resulted from the most obvious possible genetic mechanism: The H-chain genes in these lines were all rearranged while the L-chain genes were not (9, 10, 15). Later

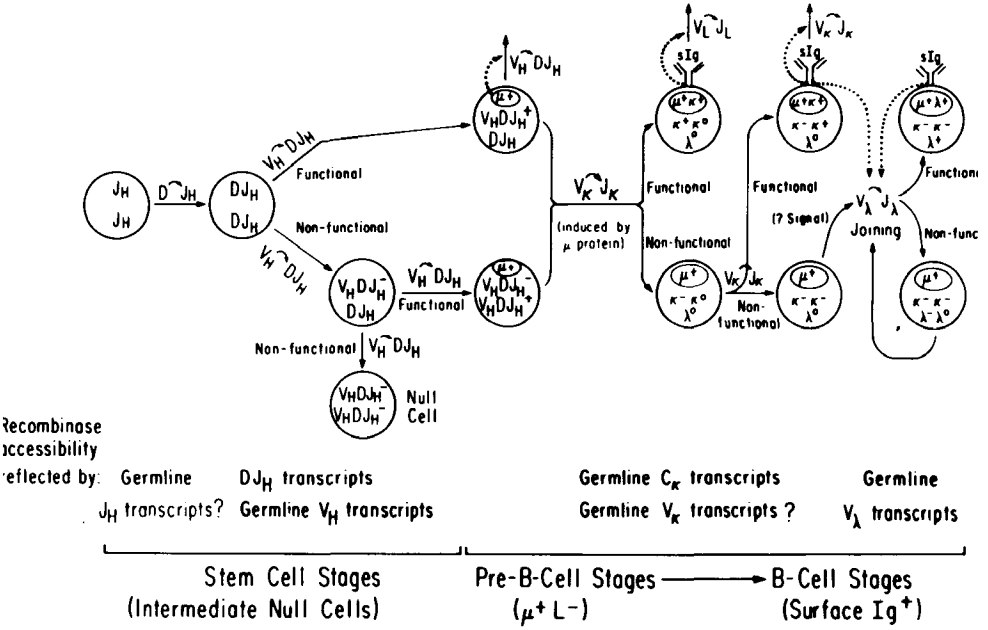

Figure 4 Schematic representation of the regulated model of allelic exclusion. A negative signal responsible for terminating further rearrangement is represented by a dotted arrow. Transcription that may reflect the accessibility of a given locus for rearrangement is indicated in the figure (see text).

investigations using normal sorted bone-marrow pre-B cells verified that ordered rearrangement of H- and L-chain genes occurs in vivo (7).

The first known step in the B-cell differentiation pathway is the joining of a D segment to a J_H segment. At least in murine B cells, this event occurs early and generally on both chromosomes, yielding a "null" pre-B cell with two DJ_H rearrangements. A tumor model for this cell is provided by the majority of A-MuLV–transformed cell lines that are derived when A-MuLV is used to transform fetal liver cells from days 14 through 18 (15, 23, 25). These pre-B-cell lines cannot produce complete μ chains, although they all express germline V_H genes and some produce Dμ proteins from their DJ_H rearrangements (51, 66). An analogous cell population was recently identified in the fetal liver (51).

The next stage of the B-cell–differentiation pathway involves the appendage of a V_H segment to the preexisting DJ_H intermediate (15, 20, 21, 23, 25). The null A-MuLV pre-B-cell lines just described are capable of actively performing V_H-to-DJ_H joining when propagated in culture. V_H-to-DJ_H joining appears to be the regulated step in H chain–gene assembly in the sense that it is allelically excluded (23). Various models had been previously proposed to explain the phenomenon of H-chain allelic exclusion, but most consistent with available data is a regulated model in which the μ protein expressed from a productive rearrangement feeds back and in some way leads to the cessation of further H chain–gene rearrangement (23, 70, 83). Thus, if a cell makes a productive V_HDJ_H rearrangement on its first attempt, the μ protein produced would prevent further rearrangement on the remaining allele, leading to a μ-positive pre-B cell that had one productive V_HDJ_H rearrangement and a frozen DJ_H complex on the second allele. If the first rearrangement were nonproductive, then the cell could continue rearrangement at the second allele. If the second rearrangement was productive, then a μ-positive pre-B cell would be generated that would have both a productive and a nonproductive V_HDJ_H rearrangement. As described above, probably no more than one third of V_HDJ_H joins are productive. Thus, it would be predicted that a significant percentage (40%—see 23) of the cells generated after V_H-to-DJ_H joining would be null cells with nonproductive V_HDJ_H rearrangements on both chromosomes. A-MuLV pre-B-cell lines derived from adult bone marrow generally seem to represent a nonselected population of cells that have just completed the V_H-to-DJ_H joining stage. Of these cells 40% exhibit the null phenotype as would be predicted, and analysis of several of these has indicated that most contain two out-of-phase V_HDJ_H rearrangements (15, 23). These null cells are different from the intermediate null cells with two DJ_H rearrangements (corresponding to the fetal liver A-MuLV transformant) in that they are presumably dead-end cells that would be lost from the

differentiation pathway; their V_HDJ_H rearrangements are fixed and cannot be replaced by normal joining events (see above).

The first evidence for regulation at the V_H-to-DJ_H joining step was based on the observation that a substantial percentage of μ-producing B-cell lines retained a DJ_H complex (23, 28, 84), indicating that rearrangement of the second allele could be blocked at the DJ_H stage. Because the only distinguishing characteristic of a productive, as opposed to a nonproductive, join is its ability to produce μ protein (both produce μ mRNA), it was argued that the μ protein itself was somehow preventing a second V_HDJ_H join in cells that had made productive rearrangements on their first attempts (70). Furthermore, assuming that approximately one in three joins are productive, the most simple form of the regulated model would predict that approximately 60% of normal B cells would have one V_HDJ_H and one DJ_H rearrangement, while the remaining 40% would have V_HDJ_H rearrangements on both H-chain alleles (23). These were approximately the numbers observed when a population of normal sorted B cells was assayed for relative content of DJ_H rearrangements (23). The frequency of productive joins in a line that rearranges its heavy-chain genes in culture (45) is consistent with the assumption that approximately one in three V_H-to-DJ_H joins results in protein production. Studies in transgenic mice have provided direct evidence that introduced H-chain genes are capable of regulating (preventing) rearrangement of endogenous loci; endogenous heavy chain–gene rearrangement was blocked in pre-B-cell lines and splenic hybridomas derived from μ-transgenic mice, although rearrangement was in some cases aborted even prior to D-to-J_H joining (85, 86).

REGULATION OF L-CHAIN VARIABLE-REGION GENE ASSEMBLY The next step in the B-cell pathway is the assembly of the L-chain variable-region genes. There has been some debate as to whether or not ordered H and L chain–gene rearrangement is due to regulation or results simply from the stochastic likelihood of forming a V_HDJ_H join as opposed a V_LJ_L join. Current evidence favors a regulated model in which μ protein plays the dual role of signaling (*a*) for the cessation of H chain–gene assembly and (*b*) for the onset of L chain–gene assembly (45). Thus, no transformed cell line has ever been observed that had rearranged L-chain genes in the absence of H chain–gene rearrangement. A few pre-B-cell lines or hybridomas that produce only light chains have been described, but most such lines analyzed in detail were found to have probably once expressed H chains but to have lost such expression due to secondary events (18). The strongest evidence in favor of the theory that expression of μ protein is a prerequisite to L chain–gene rearrangement comes from the analysis of the complete in vitro differentiation of a pre-B-cell line to the B-cell stage (45; see below).

Light-chain variable-region gene assembly is also ordered and regulated, with the sequential rearrangement of the L-chain alleles apparently terminated by a join that results in the production of a L chain that can pair with the preexisting H chain to form complete Ig. Analyses of gene rearrangement and expression at the light-chain loci provided the initial evidence for the regulated model of light-chain allelic exclusion. The high percentage (60%) of unrearranged J_κ alleles in κ-producing tumor lines or normal sorted B cells suggested that κ assembly can be frozen and is not simply the result of a high level of aberrant rearrangement (for review see 87). In fact, the level of germline κ alleles approaches the theoretical level predicted by a regulated model that assumes roughly one in three joins will result in L-chain production (87). Studies with pre-B-cell lines active in κ-gene assembly have determined that the rate of productive $V_\kappa J_\kappa$ rearrangement is indeed about 30% (19, 45). Occasionally κ producers have productive $V_\kappa J_\kappa$ rearrangements on both alleles; only one of the two κ proteins or κ fragments produced will bind to the H chain present in the cell to form surface Ig (80, 81, 88, 89).

This indicated that κ-allelic exclusion is not mediated by the production of a κ protein per se but apparently by the production of a κ protein that can combine with the preexisting H chain (80). κ producers almost never have λ gene rearrangement; while λ producers almost always have rearrangements of both κ genes (some of which may produce nonfunctional κ proteins that do not bind to heavy chain) or else deleted (80, 90–93). These results suggested that κ-gene assembly precedes that of λ genes and that productive κ-gene rearrangement also prevents λ gene rearrangement—effecting isotype as well as allelic exclusion (80, 90, 91). Separate phases of κ- and λ-gene rearrangement are further supported by the characterization of A-MuLV-transformed cell lines that actively rearrange their κ genes but maintain unrearranged λ genes (19, 45). A mechanism apparently exists whereby a cell can delete productively rearranged κ genes, allowing subsequent λ-gene rearrangement while maintaining L-chain allelic exclusion (92, 93). This deletion is site specific and usually occurs at a conserved heptamer sequence; this suggests it is mediated by the same recombinational machinery that performs variable-region gene assembly. Although such a deletion would clearly remove the repressive signal that complete Ig apparently exerts on the rearrangement process, it is not clear how such a deletion can signal for the onset of λ-chain gene rearrangement. Interestingly, this κ-deletion event appears to be much more frequent in humans, who have a much higher level of λ production than mice (93).

Recently, the regulated model of L-chain allelic exclusion has received support from transgenic mice studies. A block of endogenous κ-gene

rearrangement was found in splenic hybridomas derived from mice expressing high levels of a κ transgene (94). None of the hybridomas that secreted transgenic κ chains in complete Ig had rearranged endogenous κ genes. When a hybridoma secreted an endogenous κ chain, the κ transgene was either not expressed or not associated with an endogenous H chain; this was predicted by the regulated model of L-chain allelic exclusion. Results of another transgenic study in which the introduced κ chains were expressed at much lower levels yielded a somewhat different result: In this case, the lower level expression of the transgenic κ chain did not prevent rearrangement of the endogenous κ genes (86). This result was interpreted to mean that production of complete Ig per se was not enough to prevent subsequent L-chain rearrangement, but rather that saturation of the cellular H chain with L chain might be more important (95). A clear understanding of these results awaits demonstration that transgenic L-chain expression occurred normally at the B-cell stages when rearrangement was occurring.

The evidence presented above collectively provides strong support for the regulated model of allelic exclusion: In particular, the evidence is persuasive that in a programmed sequence of rearrangement events, μ-protein production signals for the cessation of H chain–gene rearrangement, while complete Ig turns off L-chain rearrangement. Until recently, very little data addressed the second proposed regulatory function of the μ protein—that is, its ability to trigger the onset of L chain–gene rearrangement. The recent characterization of the complete in vitro progression of a pre-B-cell line to the B-cell stage has provided evidence, within the context of the antigen-independent differentiation of a single line, for almost all the aspects of the regulated model of allelic exclusion (45). In particular, strong evidence has been obtained for the role of μ protein in the initiation of L chain–gene rearrangement. This line is now discussed in detail.

A PRE-B-CELL LINE THAT COMPLETELY DIFFERENTIATES IN CULTURE

The 300-19 A-MuLV transformant was derived from the adult marrow of outbred NIH/Swiss mice. The original isolate of this line appeared to represent the most primitive known B-cell analogue; it had DJ_H rearrangements on both J_H alleles, and no J_L rearrangements (66). Both of the DJ_H rearranged alleles are transcribed to yield Dμ mRNA, one of which encodes a Dμ protein (66). Dμ proteins are usually produced at low levels relative to that characteristic of normal μ-chain expression at this stage; this low-level Dμ production did not prevent extensive secondary J_H rearrangements in

the 300-19 line, including frequent V_H-to-DJ_H joinings (45). Approximately 20–30% of such V_HDJ_H rearrangements formed in culture were productive, providing evidence that the unselected frequency of productive V_HDJ_H joining is relatively high; progeny containing such joins accumulate high levels of normal μ chains (45). Significantly, all subclones of this line that express a complete H-chain gene have ceased further H chain–gene rearrangement but show evidence of ongoing and frequent κ-L-chain gene-assembly events; in addition, none of the many subclones that had assembled and expressed κ-L-chain genes (with one interesting exception) had done so without assembling and expressing at high levels a complete H-chain gene. Furthermore, clones that had assembled two nonproductive V_HDJ_H complexes showed no evidence of κ variable-region gene assembly, indicating that L chain–gene rearrangement does not necessarily follow H chain–gene assembly per se. These findings argued for separate phases of H- and L-chain variable-region gene assembly and strongly supported the contention that μ protein indeed serves the dual regulatory functions of signaling for the cessation of H-chain variable-region gene assembly and the onset of κ variable-region gene assembly. A clue to some of the necessary molecular determinants of this putative signal may come from the observation that one subclone that expressed extremely high levels of the Dμ chain appeared to undergo no further rearrangements of the J_H locus (it was frozen in the DJ_H state) but began rapid rearrangement of its J_κ alleles (45). Thus, Dμ proteins may contain the necessary molecular determinants to effect such regulatory events, but they are normally present at too low a level to do so. As mentioned above, it has also been proposed that L chains must be present at a critical level to effect L-chain allelic exclusion (86, 95).

The 300-19 line progresses from the most primitive pre-B-cell stage known to become a surface Ig–positive B cell, requiring no factors other than those normally found in the culture medium. This antigen-independent differentiation appears to be self-regulated, with the protein product of a given rearrangement stage initiating progression to the next stage of the B-cell developmental pathway.

PREFERENTIAL REARRANGEMENT OF V SEGMENTS BASED ON CHROMOSOMAL POSITION

The lack of randomness in the rearrangement processes thus far described seems to extend to the choice of the V_H segment used in rearrangements: In pre-B cells derived from the BALB/c mouse strain, segments from the most J_H-proximal V_H family (V_H 7183) are thought to be rearranged at an

extraordinarily high frequency (24, 11); the most J-proximal members of this family are used at the highest frequency (24). The pre-B-cell line 300-19 preferentially rearranges a different set of V_H gene segments; once again, the frequently rearranged segments map to the most J-proximal position in the strain from which 300-19 was derived (M. G. Reth, S. Jackson, F. W. Alt, in preparation). Analysis of V_H family usage in normal fetal liver reveals that early in development preferential V_H usage occurs in vivo and that this position-dependent bias for rearrangement extends across the entire V_H locus (Figure 1A, bottom; G. D. Yancopoulos, F. W. Alt, in preparation); strains that exhibit immune deficiency or develop autoimmunity seem to have normal V_H usage patterns early in development (A. Mannheimer, F. W. Alt, C. Bona, in preparation). Although very thought provoking, the observation of biased V_H rearrangement has not yet been assigned a clear role in the developing immune system. The preferentially used V_H segments in pre-B cells are not overrepresented in the mature B-cell population, raising the dilemma of how randomization of the initially skewed V_H repertoire occurs in vivo—selection (either by antigen or due to cell-cell interactions) would seem to play an important role. The complex relationship between immune response and self-tolerance may in some way depend on a limited early repertoire; limited Ab diversity early in development, which has been documented in several species, clearly would result from preferential rearrangement. Biased V_H rearrangement has not been clearly correlated with the programmed appearance of Ab specificities that occurs during ontogeny; establishing such a relationship may depend on analysis of early V_L joining preferences. Other examples of developmentally controlled expression related to the linear organization of genetic information occur with mammalian hemoglobin genes, silk moth chorion genes, and the genes in the Drosophila bithorax complex (96–98). Because it is hard to imagine how the linear organization of V_H segments could determine their rearrangement frequency if recombinase acts by joining segments that randomly collide during three-dimensional diffusion, it seems more likely that recombinase acts via a one-dimensional tracking mechanism (24, 27). This tracking mechanism would be operative at least through the stage of development when V_H segments are rearranged preferentially according to their J_H proximity.

DETAILS OF THE JOINING MECHANISM

We have already discussed many of the basic mechanistic features of variable-region gene assembly. In this section we will explore some of these features in depth, and describe a general model that can account for the imprecise joining of gene segments.

Imprecise Joining of H-Chain Variable-Region Gene Segments

The third hypervariable region of the H-chain gene spans the V_H-D-J_H junctional region (1). The hypervariability of this region is due not only to the combinatorial assortment of the many different segments that can be used to form a particular join, but also to the imprecise joining mechanisms that tend to increase diversity at the joining junctures (1). These mechanisms result in the loss and addition of bases at the site of joining, which result in the loss and generation of potential amino acid coding regions; this flexibility is an important source of antibody diversity. A multistep, nonreciprocal joining process has been proposed in order to explain the joining patterns observed between H-chain component gene segments (Figure 3A; 99). First, a double-stranded cleavage occurs at the border of the segment coding region and its flanking recognition sequence. Precise back-to-back fusion of the recognition heptamers then occurs, resulting in the deletion of the fused recognition elements and all intervening sequences between them, in the form of a circle. The coding sequences are not fused precisely: A joining event distinct from that which joins the recognition sequences allows modification of the coding sequences before joining. First, bases can be removed from the potential coding regions of both segments to be joined, and then new bases can be added at the point of joining (N-region insertion). A double-stranded exonuclease could account most easily for the loss of bases observed (99), although other mechanisms are also possible (100). It has been suggested that terminal deoxynucleotidyl transferase (TdT) could be responsible for the addition of bases to the joints (99). This enzyme has the ability to add nucleotides to a free 3′ hydroxyl group and is expressed at high levels in immature lymphocytes. Replication of the added bases and ligation of the altered segments (not necessarily in that order) must be accomplished to complete the joining process.

The recombination recognition sequences seem to be responsible for targeting the recombinational machinery to perform the proposed initial event in recombination—the double stranded cut. Although there is no direct evidence either for this targeting or for the existence of a double-stranded cut, extracts have recently been prepared that can make double-stranded cuts near J-joining signals in vitro (101, 102). Analogously, in yeast, it is clear that a sequence-specific endonuclease initiates the mating-type switch event by performing a double-stranded break (103). H-chain rearrangement occurs via an intrachromosomal deletion mechanism (Figure 3B). Sequences between D and J_H are lost in cells that have formed DJ_H rearrangements, whereas all D segments are lost in lines that have two V_HDJ_H rearrangements (23). The only evidence for the precise fusion of the

heptamer sequences during H-chain gene assembly was obtained upon examination of an aberrant D-to-J_H join that occurred via a complicated inversion process, resulting in retention of the recombination recognition elements (99). Examination of these retained elements not only demonstrated that the heptamers do indeed undergo back-to-back fusion, but also that this fusion is precise. The occurrence of base loss and base addition during the joining of component gene segments was originally defined by extensive comparisons of variable-region joins in terminally rearranged cells with the sequence of the corresponding unrearranged segments (1). A dynamic analysis of the mechanisms involved in base deletion and addition was provided by studies of A-MuLV-transformed cell lines active in V_H-to-DJ_H joining in culture (25). Comparison of the nucleotide sequence of a series of V_HDJ_H joints that were assembled in culture with that of their component V_H and DJ_H precursors allowed a precise definition of the bases added and deleted. Of the two actively rearranging lines analyzed, only one appeared to be capable of base addition; strikingly, the level of TdT activity in these two lines correlated to their ability to insert N regions, supporting the notion that TdT may be responsible for N-region insertion (25).

Mechanistic Differences Between H- and L-Chain Variable-Region Gene Assembly

The assembly of H- and L-chain variable-region genes appears to employ similar mechanisms. However, deletion of bases upon joining is more limited in L-chain joining, and addition of bases to V_LJ_L joins is rarely if ever observed. Because L-chain variable-region assembly occurs at a different stage than H-chain assembly but appears to use the same basic recombinase system (see below), it is possible there are stage-dependent levels of certain accessory enzymes responsible for base deletion/insertion. Alternatively, it is possible that there is a very strong selective pressure to maintain the size of the L-chain variable region. However, this seems unlikely since the unselected V_LJ_L joins within an introduced recombination substrate mimic normal L chain–gene joins (100).

Another difference between H chain– and κ L chain–gene assembly is that sequences lying between V_κ and J_κ are often retained in the cell after $V_\kappa J_\kappa$ joining (these sequences often include fused recognition elements; 19: 104–107). Several models were proposed to explain the retention of the intervening sequences in $V_\kappa J_\kappa$ joins; these include joining by sister chromatid exchanges (104, 105), reintegration of sequences initially deleted as a circle (104, 105), and an inverted joining mechanism (19, 106, 107). Currently, the inversion model seems to best explain the retention of intervening sequences after $V_\kappa J_\kappa$ joining (Figure 3B). According to this model the basic difference between H chain– and κ L chain–gene assembly

can be explained by postulating that, unlike V_H segments, some V_κ segments are either inverted with respect to the J_κ cluster or are located downstream to it. That inverted joining between widely separated chromosomal elements is possible was demonstrated by the occurrence of inverted DJ_H joining (99). Inverted joins between V_κ and J_κ segments within introduced recombination substrates (see below) also demonstrated that such joining is possible between these elements if they are arranged in inverted orientation (100, 108). Clearly, definitive evidence that endogenous κ genes rearrange by inversion will involve direct demonstration of the relative location and orientation of the V_κ and J_κ segments on the chromosome. In this regard, a recent analysis has described the linkage of the V_λ and J_λ gene segments in the chicken; notably, several V_λ segments were in the inverted orientation, although it was not shown that they rearrange (41). A clear case of inverted joining as a normal mechanism of joining endogenous genes has recently emerged from the analysis of V_β-J_β joining in T cells (L. Hood, personal communication).

Evidence for the Joining Model from Recombination Substrate Studies

Recently several groups have observed site-specific recombination of variable-region gene segments that were introduced as recombination substrates into A-MuLV transformants. Probably the key factor in the success of these experiments was the derivation of recipient A-MuLV-transformed cell lines that were active in Ig-gene assembly. Such experiments allow more precise definition of the molecular requirements of the joining process.

One set of studies involved the introduction of recombinant DNA plasmids containing D and J_H segments separated by the herpes simplex virus thymidine kinase (HSV-tk) gene into TK-negative derivatives of fetal liver transformants (109). Selection against expression of the HSV-tk gene (by growing the cells in the presence of bromodeoxyuridine) selected for cells that had deleted the HSV-tk gene by site-specific joining between the flanking D and J_H segments (Figure 3C, left); various criteria indicated that the joining process was a faithful replica of the endogenous process. A similar experiment utilizing T cell–receptor D_β and J_β segments (G. D. Yancopoulos, T. Blackwell, H. Suh, L. Hood, F. W. Alt, *Cell*, 44, in press) revealed that these introduced segments could be induced to rearrange at a high rate in pre-B cells (see below). A different approach was used to examine introduced κ-gene rearrangement in marrow-derived A-MuLV transformants that actively assembled endogenous κ genes in culture (108). In this case, V_κ and J_κ segments, in inverted orientation, were introduced via a Moloney MuLV–derived retrovirus vector; activation of a positive

selectable marker located on this vector required the inverted joining of the V_κ and J_κ segments (Figure 3C, right). This type of vector allowed recovery of not only the joined gene segments, but also of the reciprocally joined recognition sequences.

Several insights into mechanistic detail have been provided by these experiments:

1. They have provided direct evidence for the deletional mechanism of DJ_H joining (109) and have also verified that joining can occur between segments arranged in an inverted configuration (108).
2. They have confirmed that although the flanking heptamers are precisely joined, there is imprecision in the joining of the variable-region gene segments themselves (108, 109).
3. They have correlated the levels of TdT in the recipient cell line with N-region insertion within an introduced recombination substrate (G. D. Yancopoulos, T. K. Blackwell, H. Suh, L. Hood, F. W. Alt, *Cell*, 44, in press).
4. They have demonstrated that a precise chromosomal location is not required for recognition and joining by recombinase. In addition, these experiments have defined the minimum flanking sequences necessary for recombinase joining of introduced sequences. Thus, the sequences required for targeting and binding recombinase must all lie within these narrow regions included on the introduced constructs.

THE ACCESSIBILITY MODEL TO EXPLAIN CELL- AND STAGE-SPECIFICITY OF JOINING

As described above, the ordered rearrangement of Ig-gene segments has apparently evolved to ensure the clonal specificity of the B-cell response to antigen; similarly, ordered rearrangement appears to play a role in T-cell antigen-receptor assembly (110–112). Tissue- and stage-specific rearrangement is dependent upon the ability to differentially perform and regulate the many different segment-specific joining events. However, the recombination sequences that flank all Ig and T-cell antigen-receptor component gene segments are quite similar, with no consistent differences related to the tissue or stage specificity of rearrangement (1, 42). The similarity between the recombination recognition sequences flanking different gene families that diverged millions of years ago indicates a very important and basic functional role for these sequences; in fact it raises the possibility that these highly conserved recognition sequences may be responsible for the targeting of a common recombinase. If a common recombinase is present that can perform all the various segment-specific joining events, a higher

level of control would be required to regulate the segment specificity of its activity. Insight into this higher level of control may be provided by the pattern of expression of unrearranged/incompletely rearranged gene segments, which we have reviewed above. Unrearranged gene segments appear to become transcriptionally active just at or prior to the cell stage in which they are normally rearranged (Figure 3, bottom). This transcription may reflect an increased accessibility of these gene segments to both transcriptional and recombinational machinery; thus, a single common recombinase could be specifically targeted to make a particular type of join by regulating the level of accessibility of the component gene segments (51). For example, regulation of the "opening" of the V_H locus, as reflected by germline V_H transcription, would allow specific control of the critical V_H-to-DJ_H joining step. The μ protein resulting from a productive join would signal for the "closing" of the V_H locus, preventing further V_H-to-DJ_H joining, and would also induce "opening" of the κ locus, initiating V_κ-to-J_κ joining. Such a model for recombinational control proposes a second function for the Ig enhancer elements—"recombinational enhancement" based on control of accessibility—and predicts that similar as yet undefined control elements regulate the accessibility of the other component gene segments. This model is by no means complete. Other levels of recombinational control are obviously at work; for instance, recombinase seems to act by a one-dimensional scanning model, at least through the early stages of V_H to DJ_H joining (see above). Cooperation of "tracking" and "accessibility" mechanisms may occur.

Two recent recombination substrate experiments have supported the "accessibility model." One experiment demonstrated that the frequency of D-to-J_H joining, within a recombination substrate introduced into a rearranging pre-B-cell line, could be regulated by controlling the expression of an adjacent selectable marker gene (T. K. Blackwell, H. Suh, F. W. Alt, in preparation). A second experiment introduced a recombination substrate containing T cell–receptor β-chain gene segments into a pre-B-cell line (G. D. Yancopoulos, T. Blackwell, H. Suh, L. Hood, F. W. Alt, *Cell*, 44, in press). The corresponding endogenous segments do not normally rearrange in such lines, but the introduced segments were able to rearrange at a high rate. This rate was apparently related to the accessibility state of these introduced segments as compared to their endogenous counterparts. This experiment clearly demonstrated that a recombinase activity normally present in pre-B cells could be targeted to perform a site-specific join normally seen only in a different cell type, as would be predicted by the accessibility model for recombinase regulation.

The modes of recombinase regulation reviewed here have recently been invoked to explain site-specific recombinase control in prokaryotes (113, 114). It has been proposed that prokaryotic recombinational enhancers,

reminiscent of eukaryotic transcriptional enhancers, facilitate site-specific joining either by providing an entry site for recombination proteins or by making the substrate DNA more accessible to recombination. One-dimensional tracking models have also been the preferred way to explain the predilection of prokaryotic site-specific recombination proteins for adjacent and correctly oriented sites (115). Accessibility as a mode of control for DNA-modifying enzymes also has precedent in eukaryotic organisms. It is well known that DNase 1 and other endonucleases preferentially cleave actively transcribed DNA. It has also been demonstrated that the repair of UV-induced pyrimidine dimers, presumably initiated by an adduct-recognizing excision enzyme, occurs much more efficiently at active loci than in the genome as a whole (116). Mating-type switch in yeast provides perhaps the best characterized example of an accessibility control mechanism analogous to Ig recombinase control. The switch event is initiated by a site-specific, endonuclease-mediated double-strand break. This cut normally occurs at only one of three identical DNA sequences, the transcriptional activity of these sequences apparently determines their ability to be cut. The chromatin accessibility of these sequences, as reflected by their transcription, may determine their ability to be recognized by the initiating endonuclease (103).

Thus, DNA accessibility seems to be important as a general biological control mechanism. It has recently been invoked to explain the apparent targeting of heavy-chain class switching in certain lines to specific constant regions (117). It may also explain the tendency of certain genes (such as oncogenes) to be modified/amplified only in the cell types in which they are normally expressed at high levels (118).

PERSPECTIVES

The assembly of Ig and T cell–receptor variable-region genes involves a series of complex and highly regulated genomic recombination events. Recent studies have suggested that tissue- and stage-specific control of variable-region gene assembly is mediated by regulating the accessibility of component gene segments to a common enzymatic machinery (recombinase). However, much remains to be learned. Thus, although implicated in the control of specific gene expression in many differentiation pathways, chromatin accessibility as a means of regulation is not yet understood in any system.

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CONTENTS

Ann. Rev. Immunol. 1986. 4 : 369–88

CURRENT STATUS OF IMMUNOLOGICAL ADJUVANTS

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> "However interesting they may be for application, they are at least as valuable because of the investigations that they may generate in order to understand the intimate mechanisms . . . of the production of antitoxins."
>
> Ramon, 1926

INTRODUCTION

Sixty years ago Ramon demonstrated that it was possible to augment the antitoxin response to diphtheria and tetanus by administering vaccines with pyogenic bacteria or with various additional compounds (1). Since that time, clinicians and immunologists have sought to potentiate the immune response with adjuvants while attempting to minimize the often present side effects.

An explosion of information concerning the complexity and sophistication of immune regulation has occurred in the past decade. Coupled with presently available biosynthetic and recombinant DNA technology, this knowledge is permitting development of vaccines possessing antigenic epitopes that were previously impossible to produce. Current vaccine candidates, for example, include synthetic peptides mimicking streptococcal (2), gonococcal (3), and malarial antigens (4). However, these purified antigens are generally weak immunogens that will require adjuvants to evoke protective immunity. Thus, a corresponding surge of interest has arisen in the development of potent nontoxic adjuvants that will enhance the immunogenicity of haptenic epitopes. An adjuvant may also be needed for conventional vaccines in order to elicit an earlier, more potent, or more

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prolonged response, or in cases where antigen supply is limited or too costly to produce.

The development of adjuvants has until recently been empirical. An enormous number of compounds have been found to modulate the immune response. These compounds have been notably diverse in both substance and function, a fact that has complicated attempts to discover the unifying mechanisms of adjuvant action. The elucidation of these mechanisms has lagged behind recent advances in the understanding of the immune system.

This diversity of adjuvants has presented difficulties in their classification. Adjuvants are occasionally grouped according to their origin, be it mineral, bacterial, plant, synthetic, or host product. Adjuvants with bacterial origins have recently been purified and synthesized (e.g. muramyl dipeptides, lipid A) and host mediators have been cloned (Interleukin 1, 2), providing chemically characterized products for study.

Adjuvants have been also categorized by their proposed mechanisms of action. This type of classification is necessarily somewhat arbitrary because most adjuvants appear to function by more than one mechanism. Adjuvants may act through antigen localization and delivery, or by direct effects on macrophages and lymphocytes. The creation of an antigen depot appears to be important for the activity of aluminum compounds, oil emulsions, liposomes, and synthetic polymers. Three of these adjuvants are reviewed in the first section of this chapter. The adjuvant activity of lipopolysaccharides (LPS) and muramyl dipeptides (MDP) appears to be mainly mediated through activation of the macrophage, whereas *Bordetella pertussis* affects both macrophages and lymphocytes. We address the adjuvant actions of these three bacterial products in the second section. In the final section we discuss some very recent and speculative approaches to immunopotentiation, such as the utilization of monokines and lymphokines, and the manipulation of the antigen, carrier, and adjuvant to augment the immune response.

Several excellent and comprehensive reviews of adjuvants and their mechanisms have been recently published (5, 6, 7). We concentrate on adjuvants of bacterial origin that are naturally or synthetically derived, because notable chemical advances have occurred with these adjuvants in the last decade. Length limitations prevent discussion of many other compounds such as saponin, a widely used adjuvant for veterinary vaccines.

NONBACTERIAL ADJUVANTS

Aluminum Compounds

The first use of aluminum compounds as adjuvants was described in 1926 (8). Since that time antigens precipitated with aluminum salts or antigens

mixed with or adsorbed to performed aluminum compounds have been used extensively to augment immune responses in animals and humans. Their wide use has been summarized in an older (9) and a more recent clinical review (5). Aluminum compounds and similar adjuvants appear to work through the following means:

1. Excretion of the antigen is slowed, thus prolonging the time of interaction between the antigen and antigen-presenting cells such as macrophages or follicular-dendritic cells.
2. Immunocompetent cells are attracted to the area of injection. Aluminum particles have been demonstrated in regional lymph nodes of rabbits 7 days following immunization (10), and it may be that another significant function is to direct antigen to T cell–containing areas in the nodes themselves. Adjuvant potency has been described to correlate with inflammation of the draining lymph nodes (11).

While many studies have confirmed that antigens administered with aluminum salts led to increased humoral immunity (HI) (reviewed in 5), cell-mediated immunity (CMI) appears to be only slightly increased, as measured by delayed-type hypersensitivity (DTH) (12). Aluminum hydroxide has also been described as activating the complement pathway (13). This mechanism may play a role in the local inflammatory response as well as immunoglobulin production and B-cell memory (14).

Primarily because of their excellent record of safety, aluminum compounds are presently the most commonly used adjuvants in humans. They are, however, not without problems. Aluminum containing vaccines occasionally cause local reactions (10, 15). Although allergic manifestations are not usually a clinical problem, aluminum compounds have been also said to attract eosinophils to the area of injection via a T cell–dependent mechanism (16), to induce an IgE response if injected after antigen priming (17), and to elicit a carrier-specific cell population with helper function for IgE response (18). In addition, aluminum-containing vaccines cannot be lyophilized, thus necessitating refrigerated transport and storage. Finally, and most importantly, aluminum compounds are not always successful in inducing a better response than aqueous controls. Thus, while aluminum salts have been a sufficient adjuvant for strong immunogens that require antibody responses only to elicit protection, more potent adjuvants that elicit cell-mediated immunity are needed.

Liposomes

In 1974 diphtheria toxoid entrapped in artificial liposomes composed of lecithin, cholesterol, and stearylamine in molar ratios of 7:2:1 was demonstrated to elicit a better primary antibody response than free toxoid (19). These findings have since been confirmed by many investigators

utilizing diverse antigens. Experiments to determine whether antigen entrapped within the liposomes is a more (20) or less (21) effective adjuvant produced conflicting results. These prompted a controversy regarding the question of whether liposomes with surface-associated antigen can substitute for macrophages (22), or whether the liposomes are ingested by macrophages leading to more efficient antigen presentation (23). The charge (19), composition (21, 24), and method of preparation (22, 24) of liposomes all influence their adjuvanticity. The number of lipid layers also appears to be relevant but is dependent on the system utilized.

The mechanism of action of the enhancement of humoral immunity induced by liposomes appears to be due in part to their function as a depot for antigen (23). A physical association between antigen and liposome is a necessary condition for the potentiation of antibody response. Simultaneous injection of empty liposomes and protein antigen revealed no primary antibody response when compared with injection of the same amount of protein antigen entrapped within the liposomes (25). In addition, the augmentation of humoral immunological memory by protein entrapped in liposomes retains the T-cell dependency of native protein (25).

There must, however, be other mechanisms of action as well, because antigens entrapped in liposomes, unlike antigens injected with aluminum salts or oil emulsions, appear to elicit cellular immunity in addition to humoral immunity (26, 27). The reason for this is not clear, although it may result from antigen presentation in a hydrophobic microenvironment. Protein antigens directly conjugated to lipid moieties induce increased delayed type hypersensitivity DTH in proportion to their hydrophobicity (28, 29). This appears to be caused by an increased uptake by macrophages resulting in more efficient presentation to T cells (30). The potency of liposomes for both humoral and cellular responses can be further increased by the inclusion of bacterial lipopolysaccharide (31), lipid A (32), or muramyl dipeptide (33) within the liposomes in addition to antigen.

The anatomic distribution of liposome-entrapped antigen depends upon the route of administration. Intravenous use results in rapid delivery of antigen to the liver, spleen, lungs, and kidney. Intradermal or intramuscular administration results in a depot in the area of the injection that is slowly absorbed over a period of days to weeks (23). It may be that these differences could be utilized to induce local or systemic immunity. Liposomes containing muramyl dipeptide have been shown to localize it efficiently within the lung for the purpose of eradicating experimental tumor metastases (34). It seems likely that local specific immunity to infectious agents could be induced by similar methodology.

While further studies remain to be done regarding toxicity and efficacy, and methods need to be developed to ensure stability, liposomes are a potentially promising delivery system for vaccines.

Synthetic Polymers

A recent and novel approach to studying depot and antigen-localizing adjuvants has been the use of nonionic polymer surfactants (35, 36). Pluronic polyol compounds composed of copolymers of hydrophilic polyoxyethylene and hydrophobic polyoxypropylene in different proportions have been utilized to examine the relevance of the physiochemical presentation of an antigen on an oil droplet. Copolymers that were relatively hydrophobic promoted the retention of bovine serum albumin on oil droplets and induced a higher antibody response than copolymers that were hydrophilic (35). By manipulating the array of hydrophilic and hydrophobic copolymers in oil emulsions with antigen, it was shown that the induction of antibody responses could be dissociated from the induction of inflammation and granuloma formation (36). Large insoluble polymers with hydrophilic moieties flanking hydrophobic moieties favored chemotaxis, complement activation, and antibody formation. When the proportion of the hydrophilic and hydrophobic moieties was kept constant and the molecular weight of the polymers was decreased, inflammation was favored rather than an adjuvant effect. On the other hand, polymers with hydrophobic moieties flanking hydrophilic moieties tended to have granulomatous responses (36).

These studies demonstrate that the host response to an antigen depends upon its local presentation in addition to its concentration, and such response raises the possibility of manipulating antigen presentation for therapeutic advantage. Synthetic polymer adjuvants are, however, at an early stage of development. Further work will need to be done to assess their effect on cellular immunity. As their use has depended upon the ability to alter antigen presentation on the surface of oil droplets, they will be subject to the same concerns about toxicity as mineral oil. In addition, the in vivo metabolism of the polymers will need to be evaluated carefully before they can be used clinically. Nevertheless synthetic copolymers also appear to be quite promising candidates for future adjuvant use.

ADJUVANTS OF BACTERIAL ORIGIN

The last decade has brought significant progress in the chemical purification of three adjuvants of bacterial origin: *Bordetella pertussis*, LPS, and Freund's complete adjuvant (FCA). *B. pertussis* is of interest due to its ability to modulate cell-mediated immunity through action on T-lymphocyte populations. For LPS and FCA, the adjuvant-active moiety has been identified and synthesized, which permits study of structure-function relationships and the possibility of modifying the original adjuvant to create a more beneficial toxic-therapeutic ratio.

Bacterial Lipopolysaccharide

The adjuvant activity of LPS was first described in 1956 (37). Since then, several mechanisms have been advocated to explain its adjuvant action. Most studies have reported that it can directly activate macrophages. Its adjuvant properties may stem from its ability to stimulate the production and release of monokines such as Interleukin 1 (IL-1) described in a following section. Another mechanism by which LPS may act is through increasing or decreasing the expression of Ia molecules on the surface of macrophages (thus affecting antigen presentation to T cells). It appears that Ia expression is dependent upon the length of time following the exposure of LPS to macrophages. Short exposure times in vitro (38) or in vivo (39) lead to decreased expression of Ia, whereas longer exposure times lead to increased Ia expression (39, 40). Lipopolysaccharide also has direct effects on lymphocytes. It is a T cell–independent polyclonal B-cell mitogen (41). The relative importance of this phenomenon as a mechanism of the adjuvanticity, as opposed to the role of macrophages and monokines, has been debated extensively. Lipopolysaccharide stimulates natural antibody if administered without an antigen, and specific antibody if injected with an antigen (42). Lipopolysaccharide also stimulates cell-mediated immunity as measured by DTH (43).

Since LPS is highly toxic, efforts have been directed to decrease its toxicity by chemical treatment. Succinylated or phthalylinated LPS is 100,000-fold less toxic than native LPS but retains its adjuvanticity (44). Derived from mutants of *S. typhimurium* that have been chemically degraded to remove a phosphate group, natural lipid A reportedly maintains its mitogenicity while becoming markedly less toxic as measured by pyrogenicity and chick embryo lethality (45).

The biological properties of LPS, including adjuvanticity, have long been postulated to reside with the lipid A portion of the molecule. This postulate has now been confirmed by the production of a family of wholly synthetic lipid A compounds with multiple activities including the local Schwartzman phenomenon, pyrogenicity, mitogenicity, and lethality (46–48). The use of LPS as an adjuvant has been prevented by its high toxicity. Studies of synthetic lipid A analogues have proven, however, that the activities of lipid A can be dissociated from one another (46, 49), thus providing hope that it might be possible to construct a nontoxic lipid A with adjuvant activity. Synthetic compounds have been developed with mitogenic potency similar to natural LPS. The mitogenicity of these synthetic lipid A products appears to be inhibited by polymyxin B, similar to natural lipopolysaccharide (47). In addition, the synthetic compounds are not mitogenic for LPS-resistant splenic cells from C3H/HeJ mice (47). Study of

a family of different synthetic compounds has revealed that a phosphate group at position 1 of the reducing glucosamine and amide-bound acyloxyacyl residues are important for mitogenicity (47).

Synthetic (49A) and natural (32) lipid A maintain their adjuvanticity when incorporated into liposomes, whether the antigen is incorporated into the same liposome or is separate. This may be a possible method of avoiding the solubility problems attendant with such hydrophobic compounds. In fact, when LPS is incorporated into liposomes, cell-mediated immunity is augmented, a strategy that has been proposed to increase immunity for organisms causing intracellular infections such as salmonella species (31).

Bordetella Pertussis

Whole cell *Bordetella pertussis* vaccine possesses several immunomodulatory substances that act together to enhance the immune response. Principal adjuvant components are LPS and pertussis toxin (PT), a protein exotoxin that has been linked to the harmful effects and long-lasting immunity to whooping cough (50).

Recent studies have employed highly purified preparations of PT which allow the study of its immunomodulating activities devoid of the confounding effects of LPS contained in suspensions of killed organisms. These studies have demonstrated that PT potentiates the immune response, particularly CMI, partly through its ability to alter recirculation of T lymphocytes (6, 51). Evidence of the effect of pertussis toxin on CMI through the T lymphocyte was obtained in studies examining the ability of PT to potentiate the inflammatory response to Freund's complete adjuvant (52) and on its ability to modulate DTH responses (51, 53, 54). Intravenous injection of submicrogram quantities of PT produces intense and prolonged footpad swelling at the site of injection of FCA in normal but not in nude mice (52). Moreover, administration of PT to mice 1 or 3 days after sensitization with Keyhole Limpet Hemocyamin (KLH) and FCA greatly intensifies and prolongs DTH reactions elicited with KLH alone (53). In these experiments, pertussis toxin was shown to enhance antigen driven inflammatory responses but did not affect inflammation induced by nonspecific irritants.

In adoptive transfer experiments, recipient mice that are administered PT plus lymph node cells from KLH-sensitized mice exhibit more intense and prolonged DTH reactions upon antigenic challenge than do non-PT-treated mice (53). In addition, injection of PT into donor mice greatly enhances the ability of spleen or lymph node cells from these mice to transfer DTH responsiveness to naive recipients (54). In lymph node cells from both normal and sensitized mice, PT increases the ratio of Lyt-2-negative-to-Lyt-2-positive T cells and decreases the proportion of B cells.

This alteration in the balance of T cells in secondary lymphoid organs may contribute to the ability of PT to modulate DTH response.

Pertussis toxin possesses the ability to break DTH-unresponsiveness to high doses of sheep erythrocytes (SRBC) in mice (51). Mice injected intravenously with high doses of SRBC (10^9) are unresponsive to subsequent elicitation of DTH reactions. However, high and persistent DTH responses are observed if PT is injected on days 1 to 21 after sensitization. The restoration of DTH responsiveness is accompanied with the appearance of circulating DTH-mediating cells. In non-PT treated mice, DTH mediating cells, which transfer DTH to naive recipients, are found in the spleen but not the blood. Inhibition of the function or induction of splenic DTH suppressor cells upon PT administration is also observed. Treatment with pertussis toxin was not shown to suppress B-cell responses, however; titers of circulating antibody to SRBC in PT-treated mice primed with high doses of SRBC were equal to or higher than those of control mice primed with SRBC alone. These data further suggest that altered recirculation of lymphocytes contributes to the ability of PT to break DTH unresponsiveness, as well as to its adjuvant activity.

Suspensions of killed *Bordetella pertussis* organisms have been known for decades to potentiate antibody formation to unrelated antigens administered with them. Purified pertussis toxin enhances IgG and IgE production (55). In addition, the ability of *Bordetella pertussis* to block carrier-specific epitopic suppression of antibody responses has been shown recently (56). Epitope suppression occurs when a host is immunized with a haptenic epitope conjugated to a carrier to which the animal has been previously immunized (56, 57). A strong secondary antibody response to the carrier is produced, but IgG responses to the linked haptenic epitope are suppressed. Injection of *Bordetella pertussis*, but not FCA, at the time of carrier priming limits the induction of epitopic suppression (56). Investigations in our laboratory indicate that purified pertussis toxin can also block epitopic suppression (F. R. Vogel, C. Leclerc, M. Jolivet, F. Audibert, T. W. Klein, L. Chedid, manuscript in preparation). Epitopic suppression is mediated by discrete populations of suppressor T-cells (57). Therefore, abrogation of epitopic suppression by *B. pertussis* organisms or toxin may be mediated through diversion of suppressor-cell subpopulations from secondary lymphoid organs at the time of priming by the carrier.

Freund's Adjuvants and Muramyl Dipeptides

The combination of an emulsion of mineral oil and killed mycobacteria (FCA) is one of the most potent adjuvants known for stimulating both humoral and cellular immunity. It is, however, too toxic for use outside of

the laboratory, mainly because it contains nonmetabolizable mineral oil and because the mycobacterial elements elicit severe granulomatous reactions.

Oil emulsions alone, such as Freund's incomplete adjuvant (FIA) have been demonstrated both to localize the antigen in its initial depot (58) and, like alum, to disseminate to the lymph nodes (59), which has been thought to be more important for its adjuvant function (6). Water-in-oil emulsions made with an emulsifier such as Arlacel A have been used clinically in humans and induce an improved antibody response, compared to control preparations (reviewed in Ref. 5). Major disadvantages have been questions of toxicity concerning local granulomas and cysts (60, 61) and concerns raised by the development of tumors in mice given different mineral oil adjuvants (62). The latter resulted in their removal from use in the United States. It should be noted, however, that long-term follow-up of patients who had received FIA with influenza vaccines failed to show an increase in mortality, tumors, or autoimmune diseases attributable to the oil adjuvant (63). A final disadvantage has been that FIA does not substantially increase CMI.

Recent efforts to utilize oil-type emulsions have involved trying different types of oils such as squalane or squalene or metabolizable peanut or sesame oils with the hope of developing a degradable emulsion that is stable, effective, and not carcinogenic. More complex emulsions such as water-in-oil-in-water emulsions have also been tried in order to decrease viscosity and toxicity (64). Metabolizable lipid emulsions composed of materials such as glycerol and lecithin have been examined recently, but they have yielded conflicting results with respect to efficacy in the studies performed to date (65, 66).

Freund's complete adjuvant was developed in 1937 (67). A progressive series of biochemical studies attempting to purify the active adjuvant components gradually succeeded in isolating ever smaller compounds. Trehalose dimycolate (cord factor) was identified, purified, and found to have immunostimulating properties. The search culminated with the discovery of a tripeptide-monosaccharide (68). A family of similar compounds was rapidly synthesized (69, 70) and tested for adjuvant and other biological properties. The smallest structure possible that still resulted in adjuvant activity was N-acetylmuramyl-L-alanyl-D-isoglutamine or muramyl dipeptide (MDP). Several investigators then examined the relationship between chemical structure and adjuvanticity (71, 72). Since that time MDP and MDP derivatives have been studied intensively and demonstrated to have numerous potent, diverse, and interesting activities. While many of these may be clinically undesirable, they can often be dissociated from one another through the use of different MDP derivatives,

and this allows selection of compounds that are adjuvant-active yet nontoxic.

Muramyl dipeptide is able to replace killed mycobacteria in FCA for the induction of both humoral and cellular immunity (68, 70). It differs from FCA in that it does not induce a change in immunoglobulin levels in mice when given without an antigen (73). It is capable of inducing humoral immunity when administered with an antigen in saline (74) and induces an antibody response when given orally, even if the antigen is given by a different route (71).

The classes of antibody elicited by an antigen given with MDP depend on the method of administration. Aqueous MDP given to mice with an antigen stimulates only IgG_1 and not IgG_2 or IgM antibodies (73, 75). On the other hand, injection of an antigen with MDP in mineral oil induces an IgG_2 response in guinea pigs (73). Muramyl dipeptide given with BSA in mice has been reported to elicit an IgE response (76), although lipophilic MDP conjugated to an antigen inhibits the IgE response while enhancing the IgG response (77). Intragastric administration of MDP with antigen to rats resulted in an increased IgA response in both serum and saliva which was protective for dental caries if given with certain antigens from *Streptococcus mutans* (78). Aerosol administration of MDP combined with parenteral antigen priming induced increased IgA antibody in lung washings (79).

Muramyl dipeptides have been shown to stimulate specific humoral immunity to a wide variety of natural antigens from bacteria, viruses, and protozoa. Examples include *Brucella*, Pseudomonas toxoid, diphtheria and tetanus toxoid, influenza, hepatitis B surface antigen, herpes simplex type I and *Trypanosoma brucei*. Muramyl dipeptides have also been shown to elicit antibodies to numerous synthetic antigens, usually after conjugation to a carrier. Examples include diphtheria toxin, streptococcal M–type 24 protein, foot-and-mouth disease virus, *Plasmodium knowlesi* circumsporozoite protein, and hepatitis B virus surface antigen (see Ref. 80 for review).

Hydrophilic muramyl dipeptides administered in saline alone do not appear to augment CMI markedly as measured by DTH (12). On the other hand, they markedly increase cellular-mediated immunity if administered with mineral oil as FCA (12), or if administered in liposomes (81). Cell-mediated immunity is also enhanced if the MDP derivative is made lipophilic by the addition of glycerol mycolate to the peptide (82). The incorporation of lipophilic MDP derivatives into liposomes yielded even more potent cell-mediated immunity (83). Muramyl peptides administered in oil have been used to elicit antitumor effects (84) and to induce autoimmunity in experimental models (85), both of which reflect specific

cell-mediated responses. Desmuramyl derivatives of MDP that contain diaminopimelic acid but lack the sugar moiety can also enhance HI and CMI (86) but are unable to reproduce all of the activities of FCA, such as the induction of autoimmunity.

When large doses of MDP are given prior to the antigen instead of with it, the humoral and cell-mediated responses are reduced instead of enhanced (87, 88). This appears to be due to the generation of suppressor T cells (88, 89) that are present in the spleen but not the lymph nodes (90).

The precise mechanism of action of adjuvanticity is poorly understood and multivariable. Muramyl dipeptide has a direct effect on lymphocytes. It has been reported to enhance carrier-specific helper-T-cell function (91) and to increase mitogen-induced thymocyte proliferation (92). It is also mitogenic for B lymphocytes (93) and can induce primary immune responses in T cell–depleted splenic cultures (94). It is likely that the monocyte-macrophage system plays a major role through the development of a local inflammatory response and through the production of monokines. Muramyl dipeptide may also stimulate better antigen processing of antigen by stimulating phagocytosis (95), although there is no direct evidence that MDP augments antigen presentation by the macrophage. Antigen presentation and T-cell interactions have been described to correlate with the level of expression of Ia-molecules on the macrophage outer membrane (38, 96), and several adjuvants including LPS and FCA (40) have been reported to stimulate Ia expression. The expression of macrophage Ia has been reported to be unaffected by MDP (40), although preliminary experiments in this laboratory suggest that there may be some augmentation of Ia expression in splenocytes. Muramyl dipeptide reportedly increases macrophage adherence and spreading, stimulates chemotaxis, inhibits macrophage migration, and increases production of prostaglandin and collagenase (reviewed in 97). All of these likely contribute to an amplification of the response. Direct metabolic activation of macrophages has also been demonstrated by MDP-induced stimulation of glucose oxidation (98). Another mechanism of action of MDP may be through the release of IL-1. Other mediators that may be important include B-cell growth factor, fibroblast-activating factor, colony-stimulating factor, slow-wave-sleep factor, and macrophage-inhibiting factor. These factors probably affect specific humoral and cellular immunity through complex interactions with each other and their target cells.

Pharmokinetic studies have shown that aqueous MDP is cleared extremely rapidly from the bloodstream. This suggests that it must either act immediately at the cellular level or that minute amounts are effective for the stimulation of humoral immunity. Injection of aqueous ^{14}C-labeled MDP intravenously, subcutaneously, or intragastrically into mice results in

greater than 90% of the label appearing in the urine after 2 hr. When ^{14}C-MDP was given in mineral oil, 45% of the dose remained at the injection site at 24 hr, and there was 80-fold more radiolabel in the draining lymph nodes (99). The slower clearance and localization in draining lymph nodes following administration in oil may be a significant reason that this method of administration leads to much greater cellular immunity, perhaps by increasing the duration and concentration of antigen and MDP in the T-dependent areas of the lymph node.

The use of MDP derivatives clinically will depend on their toxic-therapeutic ratio. Native MDP has untoward side effects that prevent its use in humans, at least for vaccines. This is probably in part due to the production of IL-1-like molecules by macrophages. The process of screening a large number of MDP derivatives has revealed several compounds that are adjuvant-active yet not pyrogenic. One of these, murametide (NAcMur-L-Ala-D-Gln-OCH_3) induces macrophages to produce endogenous pyrogen but simultaneously inhibits its fever-producing activity by mechanisms presently not understood (100). Another non-pyrogenic adjuvant-active derivative is murabutide (NAcMur-L-Ala-D-Gln-α-n-butyl-ester), a compound which lacks many of the undesirable properties of MDP (101). One reason for this selectivity may be that murabutide induces macrophages to produce lymphocyte-activating factor, but not endogenous pyrogen (102). Animal studies have shown that murabutide has minimal side effects (103), and human studies have shown that it augments antibody responses to tetanus toxoid (104) and to a natural fragment of streptococcal-M protein (105).

FUTURE APPROACHES FOR IMMUNOPOTENTIATION

There are several other possibilities on the horizon for increasing the immune response. It may be that the idiotype network can be beneficially exploited. This approach has been shown to enhance the antibody response to hepatitis B surface antigen (106). Another approach currently being developed is the use of vaccinia virus as a vector to enhance HI and CMI to single and even multiple antigens (107). This topic is addressed in detail in another chapter. Other possibilities include the use of monokines or lymphokines, or manipulation of the antigen, carrier, and adjuvant to achieve a desired response.

Monokines and Lymphokines as Adjuvants

As noted above, one probably mechanism of action of MDP and LPS is through the activation of macrophages with release of monokines and

consequently lymphokines. A large number of immunomodulating factors have been described and it is likely that many remain unknown. For Interleukin 1 (IL-1) and Interleukin 2 (IL-2), DNA recombinant techniques have resulted in purified products. It is theoretically possible that one or the other could have future roles as adjuvants.

One major activity of IL-1 is its role in augmenting proliferation of thymic lymphocytes when in the presence of a mitogen. This activity (called LAF activity for lymphocyte-activating-factor activity) may well correlate with in vivo adjuvanticity. Partially purified IL-1 from activated macrophages that has LAF activity has been demonstrated to increase secondary IgG responses to BSA when administered 1–2 hr after the priming dose (108). IL-1 contained in macrophage supernatants has numerous diverse effects including fever and the production of acute phase reactants (109). Therefore, crude preparations of IL-1 are too toxic for adjuvant use. There is, however, evidence that their properties can be dissociated. Supernatants harvested from macrophages activated by a nonpyrogenic derivative of MDP have LAF activity but no endogenous pyrogen activity (102). Three groups have recently described the cloning of IL-1 (110–112), thus making available purified products that can be characterized and studied in abundant quantities. It appears at the present time that there are at least two different molecules (labeled IL-1 α and IL-1 β) with LAF activity (112). As with the other adjuvants, the potential clinical use of recombinant IL-1 as a clinical adjuvant will be dependent upon its toxic/therapeutic ratio. The recombinant molecules and their future derivatives should permit further investigations regarding the nature of IL-1 receptor sites, in addition to the development of nonpyrogenic compounds with adjuvant activity.

The multiple activities in vitro and in vivo of IL-2, a T-cell growth factor, have recently been reviewed (113). It has been suggested that IL-2 is induced, at least in part, from IL-2-producing T cells in the combined presence of IL-1 and antigen. The released IL-2 then activates effector T cells, which modulate CMI (114). It thus may have potential for use as an antitumor agent and for augmenting immunity in patients with immunodeficiency (113, 114). The recent cloning of IL-2 (115, 116) should also permit conclusive investigations as to its future role as an adjuvant.

Manipulation of the Antigen, Carrier, and Adjuvant in Induction of Specific Immunity

Experiments 20 years ago demonstrated that low-molecular-weight antigens, coated onto inert support substances such as acylic plastic particles (117) or bentonite (118), induce increased primary antibody responses. More recently, it was shown that making an antigen more hydrophobic

enhances cell-mediated responses to the modified antigen (28, 29). One approach that has been employed to enhance the immunogenicity of small synthetic peptides has been to couple the peptide to itself to create a polymeric antigen. This has been done successfully to increase antibody responses to a synthetic peptide of streptococcal M24 protein (2). A more elegant approach has been to couple several different synthetic peptides together to create a larger polyvalent molecule capable of eliciting greater responses to each peptide than the responses elicited by the individual peptides alone (119). With this strategy each peptide functions as a carrier for the other peptides. It is also possible to couple the antigen directly to the adjuvant itself. Muramyl dipeptide conjugated directly to a protein antigen induces a greater immune response than the protein alone (120). Another interesting manipulation is to administer an antigen combined with specific IgM. This technique enhanced the IgG response and increased survival in a murine malaria model (121). An increase in adjuvanticity has also been obtained by mixing antigen conjugated to MDP with anti-MDP monoclonal antibodies, thus creating a yet larger complex with exposed Fc fragments on the complex. Complexes with $F(ab')_2$ but without Fc fragments were less active (122). The exact mechanism of this phenomenon is unclear although it is perhaps relevant that MDP-anti-MDP complexes enhance macrophage activation 1000-fold compared to MDP alone (123).

Early studies of haptenic antigens demonstrated that conjugation of small antigens to carriers increases their immunogenicity. While efficacious in the laboratory, this approach applied to vaccines may lead to two problems. The repeated use of a carrier could induce hypersensitivity to the carrier. In addition, the administration of a vaccine epitope on a carrier to which the host has been previously exposed, such as tetanus toxoid, may lead to suppression of the new epitope (56). The recent description of totally synthetic carriers successfully conjugated to synthetic viral proteins (124) demonstrated that it may be possible to synthesize multiple different carriers for future vaccines. Another possible approach may be to incorporate *B. pertussis* toxin together with the hapten-carrier conjugate. As noted above, this adjuvant has the potential to overcome epitopic suppression. Efforts should be directed toward improving this activity and finding other less toxic compounds.

CONCLUSION

In conclusion, it should be noted that most of the compounds described in this chapter have a wide variety of activities ranging from immune suppression to the mediation of potent humoral and cellular immunity. The muramyl peptides are a good example. The expression of their properties

depends upon their chemical structure and the mode and timing of administration. It seems unlikely that there will evolve a single adjuvant for all uses. Each adjuvant and its derivatives has advantages and disadvantages. Through adept manipulation of these compounds in the future we may be able to select desired properties while avoiding undesirable ones.

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CONTENTS

Ann. Rev. Immunol. 1986. 4 : 389–417

IMMUNOGLOBULIN A: Strategic Defense Initiative at the Mucosal Surface

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INTRODUCTION

IgA is the predominant immunoglobulin in the external secretions of humans. Specific IgA-producing cells home to mucosal epithelia throughout the body to provide humoral protection as a frontline defense against pathogens borne in aerosols, the environment, and the diet.

Much of the recent research on IgA has been directed toward three central questions: (*a*) What are the factors that account for the localization and regulation of IgA-producing cells at the mucosae; (*b*) What is the nature of the mechanism that selectively transports secretory IgA to the mucosal lumen; and (*c*) What are the physiologically important roles of IgA, both in blood and in the exocrine environment. Regulation of IgA production is an extensive topic in itself and will be touched on here only briefly. We concentrate on the fate and function of IgA after synthesis and try to assess which of its experimentally described properties are essential for IgA to contribute to the well-being of the host.

SYNTHESIS AND STRUCTURE

Lymphocyte Traffic and Immunoregulation

IgA is produced by cells of the B-lymphocyte lineage, both in the peripheral and mucosal lymphoid tissue. The functions of IgA in blood are poorly

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understood, and few studies have focused on the factors that regulate circulating IgA levels. Mucosal IgA production, on the other hand, has been the subject of more intensive investigation (1, 2).

Most B lymphocytes at the external mucosae are dedicated to IgA synthesis and form a recirculating pool of cells that home preferentially back to mucosal sites (3–5). In the small intestine, these cells are enriched in Peyer's patches. Antigens present within the small intestine are sampled by a specialized type of cell, termed the M cell (6, 7), located over each Peyer's patch and bordering on the intestinal lumen. The antigens are transferred to an environment of immune cells, accessory antigen presenting cells, and regulatory T cells inside the patch (8, 9). After antigen stimulation, precursors of IgA plasma cells migrate via the lymphatics to blood, the spleen, and the liver; then they either return to the gut or localize at distant mucosal sites (10).

Several factors have been postulated to explain the accumulation of potential IgA-producing cells at these specialized sites. The mucosal environment, rich in microbial antigens and mitogens, is known to influence the immune specificity of lymphocytes in mucosal tissue (11). Chronic antigenic stimulation superimposed with modulation from neighboring T cells may result in the commitment of naive B cells towards IgA synthesis (9). This assumes that cells early in the B-lymphocyte lineage are isotypically pluripotent. An alternative view would be that a special subpopulation of B lymphocytes becomes committed to IgA production long before IgA is even expressed by these cells. Stimulation would have the effect of hastening the cells of appropriate immune specificity along their precommitted path.

T Cells and Soluble Factors

Regulation of IgA production by T cells has been studied by several groups of investigators. Kawanishi et al (12, 13) have isolated T-cell clones from mouse Peyer's patches. These clones act on lipopolysaccharide-stimulated B cells bearing IgM, but not IgA, on their surface: The B cells differentiate and begin expression of surface IgA, presumably becoming direct precursors of IgA secreting cells.

T cells have also been described that act at a later stage of differentiation. These cells bear receptors for the Fc region of IgA and are thus classified as T_α cells. They act on surface IgA–positive B cells, either to promote or to suppress IgA secretion (14–16). The size of the T_α-cell population increases in IgA myeloma (17). T_α cells have also been described that can suppress secretion and growth of IgA-producing tumor cells (18). Recent evidence suggests that the T-cell signals for help or suppression are conveyed by soluble factors capable of binding to IgA (19, 20).

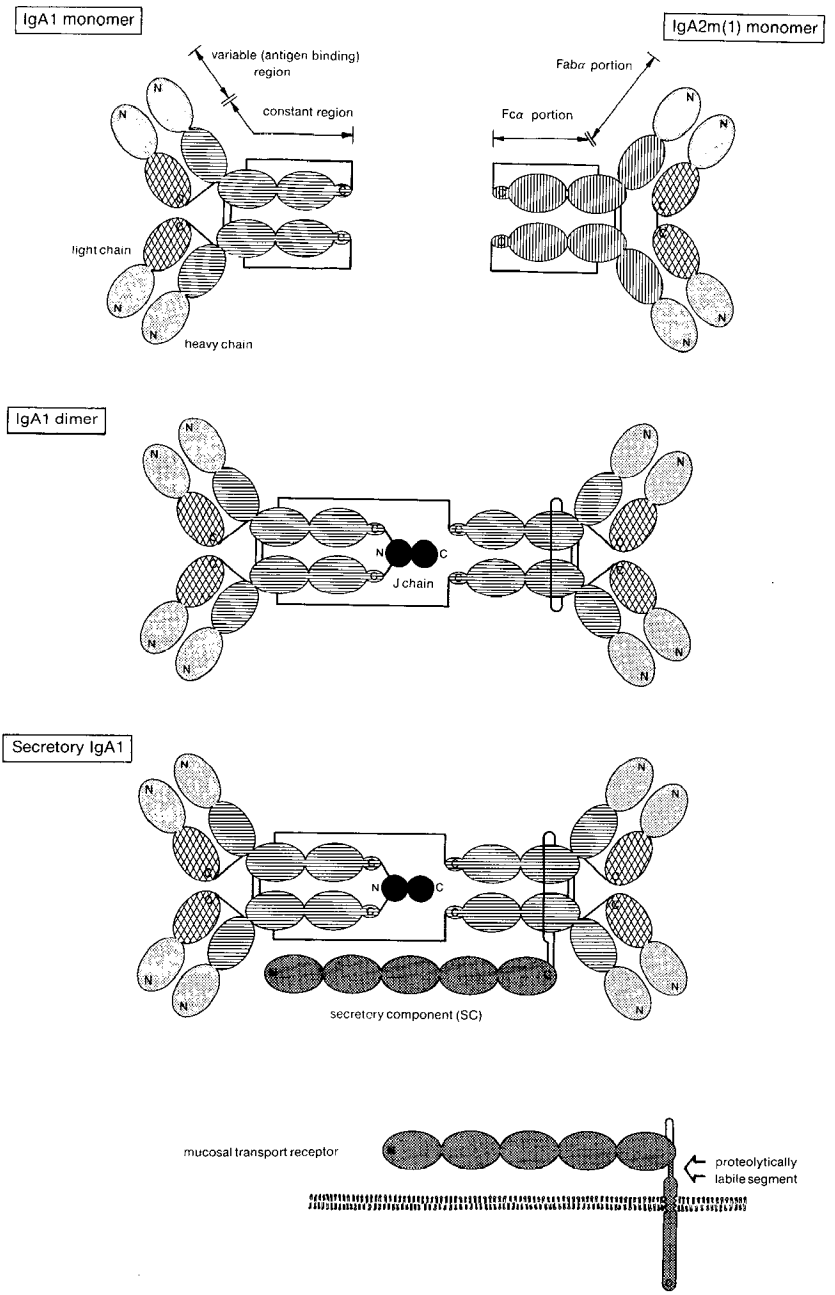
IgA Structure

Some potential synthetic products of an IgA plasma cell are illustrated in Figure 1. Like the other immunoglobulin classes, IgA is made up of four-chain-monomer subunits with two antigen combining sites oriented with two-fold rotational symmetry. Several distinctive features are probably important for IgA to function as a secretory antibody. One is the ability of IgA monomer subunits to polymerize. IgA and IgM heavy chains both possess a special C-terminal extension containing an extra cysteine, which can participate in cross-linking monomer subunits together. As a late event in synthesis, J chain (an unrelated ~15,000 M_r peptide) initiates the polymerization reaction and becomes incorporated into the molecule (reviewed in 30). When this occurs, the product of an IgM-secreting cell is pentameric; the product of an IgA-secreting cell is usually dimeric, with trimers, tetramers, and pentamers produced in decreasingly small amounts (31). Polymer formation is essential for binding to the mucosal transport receptor and increases the avidity for antigen. It also directly increases the resistance of IgA to proteolytic digestion (32).

The number of active genes (or subclasses) for IgA is different among different species. The mouse has only one, but the rabbit apparently has at least three (33). There are two subclasses in man, IgA1 and IgA2; the most notable difference between them is the hinge region. Unlike IgG heavy-chain genes that have separate hinge exons, the hinge region of the IgA heavy chain is encoded at the beginning of the exon for the middle constant region domain (Cα2). In IgA1, the hinge includes a duplicated eight amino acid, proline-rich sequence (23) that acquires five *O*-linked oligosaccharides. In IgA2, this region is replaced by a shorter stretch of consecutive proline residues (24). There are two allotypic variants of IgA2—A2m(1) and A2m(2)—of which the former appears to be a recombinant between IgA2m(2) and IgA1 (34).

The immunoglobulin hinge is generally a labile site for proteolysis, but the amino acid composition of the IgA hinge makes it an unsuitable substrate for many proteolytic enzymes. Specialized bacterial proteases have been described that cleave the IgA hinge exclusively at a single site (35, 36). Divergence of the length and composition of the hinge region of the two IgA subclasses may have been the consequence of selective pressures created by microorganisms secreting proteases with different specificities (34, 35).

The IgA in human blood is ~80% monomeric and ~80% of the IgA1 subclass. At synthetic sites in the mucosae, ~40% is IgA2 and ~90% is polymeric (37). It is not entirely clear whether this reflects a proportional difference in synthetic products of cells contributing to these two pools or



whether the ratios detected in blood are partly biased due to different clearance rates. There is no good correlation between exact serum IgA1 : IgA2 ratios and serum monomer : polymer ratios amongst different individuals (38).

TRANSPORT

Beyond antigen clearance, the functions of IgA in blood are limited. Its primary physiological role is played in the milieu of the secretory mucosa, where it is the predominant immunoglobulin class. However, the epithelial cells of the mucosa form a tight barrier between the site of IgA synthesis and the mucosal lumen. Consequently, a specific transport mechanism has evolved to conduct each IgA molecule through the interior of the epithelial cell towards its appropriate destination in secretions.

The first clue to the nature of this mechanism came with the demonstration that IgA recovered from secretions bore an additional $\sim$70,000 M_r polypeptide, secretory component (SC) (39, 40). Subsequently, immunohistology revealed no evidence of SC within the immune cells synthesizing the

Figure 1 The covalent structure of human monomeric, dimeric, and secretory IgA. Each large elliptical section represents a double β-pleated sheet 11,000–14,000 M_r with one or two internal disulfides, a domain in the immunoglobulin superfamily. IgA monomers are stabilized by both covalent and noncovalent forces, but homologous association into IgA dimer relies on disulfide bonding exclusively. Secretory component, a polypeptide of five immunoglobulin-like domains (21, 22), is generated by hydrolytic cleavage of the mucosal transport receptor on epithelial cells. Since SC binds only the polymeric form of IgA, it is likely that it interacts noncovalently with two monomer subunits simultaneously.

Disulfide bonds bridging different polypeptide chains are indicated by solid lines (based on Refs. 22–29). In IgA1, the light chains are linked covalently to the heavy chains. When the corresponding cysteine is missing from the heavy chain, the light chains link to each other [IgA2m(1)] or to the chain through an alternative residue [IgA2m(2); not shown]. The bond in the monomer linking the middle constant domain and the end of the molecule is depicted based on data from Ref. 25. This disulfide is permissible, because the second cysteine is the penultimate residue on a 19–amino acid extension to the heavy chain, which could fold back across the molecule within range of the preceding domain. However, Yang et al (26) have conflicting evidence that suggests that both cysteines link symmetrically to the opposing heavy chain.

The disulfide bonds drawn between IgA monomer subunits, J chain, and SC have been adapted from the proposal of Mestecky (27) as a likely arrangement based on currently available data. It has been proposed instead that J chain spans the two monomer subunits (28). The data are equivocal, however, and we regard this matter as unresolved. SC is bonded to the penultimate heavy chain domain of only one monomer subunit (29) even though two disulfide bonds are formed (22). The arrangement shown is a partly symmetric construction consistent with this data, but it is alternatively possible that SC links to the second monomer subunit through the heavy-chain C-terminal tail section.

IgA heavy chain, but SC was detected in the neighboring cells of the epithelium. Part of the activity was localized near the basolateral membrane facing the IgA source, and this implied that SC-IgA coupling was an early event in the transport process. Unfortunately, studying antibodies in secretions is significantly less appealing than studying antibodies in plasma. The investigation of secretory IgA (and how it became secretory IgA) received less attention than other aspects of the humoral immune response, until convenient experimental models were established.

Experimental Models for Studying IgA Transport

Analysis of tissue sections has demonstrated IgA transport at mucosal sites throughout the body, ranging from the gastrointestinal and respiratory tracts to ocular tissue, minor salivary glands, the urinary tract, the uterus, and the skin. With the one exception noted below, these sites are segregated from the circulating antibody pool by a basement membrane. Consequently, almost all the IgA in secretions is from local synthesis, with a minimal contribution from blood (41–43). An advantage of this is that levels of secretion can be regulated locally to respond to physiological needs, reflecting the estrous cycle (44) or the presence of infection.

To follow the transepithelial IgA pathway in action, researchers have developed several approaches. Nagura, Brown, and colleagues have described a human colon carcinoma line (45), subsequently cloned and selected by Mostov (46) for high levels of SC expression. Kraehenbuhl and coworkers have used the lactating mammary gland of the rabbit (47). Another model became available when Graham Jackson (on sabbatical, with Jean-Pierre Vaerman) demonstrated the transport of IgA antibody from blood to bile in the rat. Word of this finding was spread at a local meeting, and publication of the phenomena emerged from a number of groups (48, 49). Subsequently, we developed a liver perfusion technique to study hepatobiliary IgA transport in vitro (50). An important benefit of the rat liver model is the ease of supplying ligand to the endocytic face and collecting from the secretory face, while the responsible cell is still in a physiological context. Studying the cell biology of IgA transport in any of these systems has revealed features common to all mucosal sites.

Features of the Hepatobiliary IgA Pathway

Using the rat liver model, the kinetics of IgA transport across the cell have been determined precisely. Essentially all receptor-bindable IgA is cleared from blood as soon as it can access an appropriate receptor in the liver (51). The hepatocyte was identified as the responsible cell type by demonstrating specific binding of IgA to suspension cultures of isolated cells (52, 53). When hepatocytes are allowed to form monolayers, IgA can be seen entering the

cells through an endocytic process (54, 55). After endocytosis, the IgA is packaged in small ~100 nm vesicles, which are translocated across the cell to deliver their cargo into the bile canaliculus (56–58). This process takes ~20 min; the limiting step is perhaps the fusion of the vesicle with the canalicular membrane. All hepatocytes in the liver appear to be equivalent in their ability to transport IgA.

MOLECULAR WEIGHT–SIEVING RESTRICTION There is an access restriction, perhaps conferred by the hepatocyte's surface architecture, that limits the size of ligands suitable for transport. IgA dimers are cleared from blood with a half-time of about 4 min, but trimers and tetramers take progressively longer, with sufficient impairment of the tetramer to prevent a proportion of transportable ligand from ever reaching bile (59). IgM is too large to bypass the molecular weight restriction in sizeable amounts (50, 60) and remains in blood. Even IgA dimer may be restricted by the sieving mechanism, because the polymeric (Fc)α fragment is taken up significantly faster than the native molecule, approaching the kinetics of ligands cleared from the circulation on their first pass through the liver (59).

PHYLOGENY Blood to bile transport of IgA is efficient in only three of the species studied to date: the rat, the rabbit, and the chicken (Table 1). In all others (including, surprisingly, the mouse), <5% of a dose of polymeric IgA finds its way to bile within 3 hr of injection. Several additional features distinguish the second group of animals from the first: one is the relative persistence of polymeric IgA in the circulation—ranging from several hours in the mouse to several days in primates. For these animals, no short-term secretory sink has been detected for the bulk of labeled IgA polymer injected intravenously (43, 66). The balance of evidence provides a second correlating feature: In animals without a hepatobiliary IgA pathway, there is no easily detectable staining for SC in the hepatocyte (74, 75).

Physiological Role of the Hepatobiliary IgA Pathway

Since IgA is recognized by the liver through its Fc region, the transport system is blind to the antigen specificity of the immunoglobulin and to whether the antigen-binding sites are filled. By constructing complexes in antigen excess, IgA can be induced into the role of secretory carrier for small proteins (61, 72, 76, 77). This has provoked the suggestion that the hepatobiliary pathway exists as an important natural route for the disposal of bacterial antigens (78).

We believe that immune complex deposition is not the primary purpose of the hepatobiliary IgA pathway, for a number of reasons. The first is the molecular weight–sieving restriction. Only complexes formed with a single IgA polymer and a low molecular weight antigen are suitable: Antigens

Table 1 Phylogeny of the hepatobiliary IgA transport pathway[a]

Test species	pIgA source	Technique	Transport intact to bile (% injected activity)	Reference
GROUP I				
Rat:	Rat	Fistula	40–75%	(48, 49, 51)
	Rat, mouse	Isolated liver perfusion	20–40%	(50, 61)
	Affinity-purified human	Fistula	65–85%	(62)
Rabbit:	Rat, rabbit	Fistula	35–70%	(63, 64)
Chicken:	Rat, human	Dual fistula	30–50%	(65)
GROUP II				
Nonhuman primate:	Human, rat	Fistula	<1%	(66)
Human:	Human	T-tube sample	<1%	(67, 68)
Guinea pig:	Human, rat	Fistula	<5%	(69, 70)
Dog:	Dog, human	Fistula	<1%	(43)
Sheep:	Sheep, human	Fistula	<5%	(64, 69)
Mouse:	Mouse	Gall bladder sample	<5%	(71, 72)
	Mouse, rat	Fistula	1–4%	—[b]
Fish (sheepshead):	Fish	Gall bladder sample	<5%	(73)

[a] Transport of intravenously injected radiolabeled polymeric IgA (pIgA) in short-term collection studies (generally ≤ 3 h). The species are divided into two categories: Group I animals rapidly transport most or all receptor-binding IgA to bile; Group II animals transport only a small fraction of an administered dose within 3 hr, even though this transport may be demonstrably specific.

[b] A limited unpublished study (J. M. Schiff & Y. Sung) in three inbred mouse strains. Other unpublished work from T. E. Koertge & J. E. Butler suggests higher transport levels in Swiss mice if collection times are extended to 24 hr.

the size of thyroglobulin are virtually excluded from transport (60, 61). Complexes formed with reasonably large viruses or larger particles, or those formed at antibody equivalence or excess, would be ineligible for uptake. The second is the phylogeny of the hepatobiliary IgA pathway: There are only three known species in which hepatobiliary IgA transport is reasonably efficient, but surely the proper disposal of immune complexes is vital to all species. The third is that particles able to bind the receptor on the hepatocyte in a polyvalent way may be diverted from the bile excretion pathway for degradation within the cell (79). More likely, the ability of IgA to carry antigen to bile in a few experimental situations is just a fortuitous consequence of the properties of the system. For bacterial antigens, the amount actually reaching bile in the mouse is tiny compared to the amount initially present in blood (78). By far the predominant disposal route for IgA-containing immune complexes is through other mechanisms, where uptake is initiated through receptors distinct from those involved in mucosal transport.

Manning et al (80) have recently completed the bookkeeping on the source of endogenous IgA for hepatobiliary transport in the rat. Considerably more IgA flows out of the rat thoracic lymph duct than is the case in man, and this ultimately contributes most of the IgA transported from blood to bile. This finding supports the contention that the IgA pathway in the liver salvages IgA that has been synthesized in the gut but then escaped into blood, perhaps due to inadequate local transport. This salvage mechanism may actually provide a significant proportion of the humoral immunity in the intestine of the rat. There is evidently no requirement for this pathway in most species, possibly because local transport in the intestine is sufficient to accommodate the entire synthesizing capacity of the surrounding IgA-producing cells.

NO BLOOD-TO-BILE IgA TRANSPORT IN MAN When cholestasis is induced artificially in rats, there is a 10–20-fold increase in circulating IgA levels (43, 81, 82). Similarly, during liver dysfunction in man, there is a 2–3-fold increase in the level of circulating polymeric IgA (83–86). Thus, early projections suggested a hepatobiliary-IgA-transport pathway in humans. However, it turned out that in man and other primates, the hepatobiliary transport of IgA is so low as to be virtually nonexistent (66–68). In our hands, $<0.5\%$ of an injected dose is transported to bile within 3 hr of injection, apparently by an SC-independent mechanism and with no apparent preference for polymer over monomer (66). The small increase of circulating IgA in man during liver disease probably has a number of contributing factors, including: (*a*) increased synthesis of IgA by an abnormal mucosa; (*b*) decreased integrity of the intestinal mucosa causing

influx of IgA into the circulation (87); (*c*) impaired clearance of IgA, IgA-containing immune complexes, and free antigen by the liver for catabolism (85, 86, 88); (*d*) interference with hepatic localization of IgA immunocytes and diversion to systemic lymphoid tissue. Abnormal circulating IgA may be concomitant with IgA deposits in the renal mesangium (89).

Using a jejunal perfusion technique in situ, Jonard et al (90) showed that > 30 mg of IgA is secreted directly per 40 cm of human jejunum every hour. Of this IgA 98% is synthesized locally. High local transport in the intestine with negligible expression of the hepatobiliary salvage pathway in man illustrates that marked differences in the design of physiological function in different species can achieve the same end: provision of humoral immunity to the gut.

Secretory Component (SC) as the IgA Transport Receptor

Proof that a membrane-fixed analogue of SC played the part of receptor for IgA transport into secretions has come from three recent demonstrations. The first was that affinity-purified IgG antibodies against SC can also undergo limited transport across the cell by attaching to cell-surface receptors via their antigen-combining site (50, 91). The second was that among cloned colon carcinoma cells, the only ones capable of binding IgA were those that could be fluorescently stained for SC (92). The third demonstration involved preparation of two populations of human IgA polymers from a myeloma source that differed > 5-fold in their capacity to bind SC; however, the two populations were indistinguishable by all other biochemical criteria examined (93). When injected into blood, only the SC-binding fraction could be transported to bile efficiently, even though both populations were taken up rapidly by the liver.

SELECTION FOR IMMUNOGLOBULIN POLYMERS Implicating SC directly in the transport process brought with it a history of understanding obtained from SC-immunoglobulin interactions observed with isolated proteins. Free SC, isolated from sources such as colostral whey, binds the Fc region of polymeric IgA or IgM tightly, but not IgA monomer or immunoglobulins of other classes (94, 95). Transport through the liver reflects this specificity exactly (50), except for the additional molecular-weight-sieving constraint described above that discriminates against IgM and larger IgA polymers. Since IgM can partly replace IgA as the secretory immunoglobulin in IgA-deficient individuals, it is possible that this sieving effect applies less rigorously to locally produced antibody at sites outside the liver. The benefit of being able to transport IgM antibody in normal circumstances is obvious—it would provide humoral immunity to secretions in the primary immunological response before class switching occurs.

A perplexing issue is why the transport process should select so stringently against IgA monomers, when apparently any polymer will do. J chain is the only peptide that is common and exclusive to both IgM and IgA polymers. Is J chain the binding site for SC? The answer to this appears to be no: IgA polymers can be isolated that have a full complement of J chain but that do not bind SC (93). In unpublished work, we have found that using subtilisin to digest away the exposed portion of J chain from IgM does not necessarily destroy the SC binding site. Polymer selection is therefore a deliberate feature of the transport system. This feature has been long preserved in evolution; even the antibody present in fish bile is dimeric (73).

The transport system provides a second level of polymer selection, since most IgA synthesized in the mucosa is already in the polymeric form. A likely corollary is that polymeric structure is essential for IgA to perform its function after secretion. This is in contrast to IgG, the principal immunoglobulin in blood, which can satisfactorily bind antigen and exercise its effector functions without prior association of immunoglobulin monomers. It likely reflects the difference in the way circulating and secretory antibodies perform their respective duties in deactivating potential pathogens.

HIGH AFFINITY BINDING SC binds IgA first by noncovalent interaction; then disulfide bonds form subsequently, as shown in Figure 1. These steps can be unlinked by performing the binding reaction at 4°C (96). The originally published association constant was $\sim 10^8$ M^{-1} (95), which indicates only a moderately strong association that should be readily reversible. Similar values have since been published by a number of labs, but we now know that the original binding studies were interpreted in error (97). Offsetting protein heterogeneity and separation problems in this reaction can conspire to generate an apparently linear Scatchard plot with an artificial slope. Isolated rat liver membranes bind virtually all active IgA added to the medium up to its saturation point, even when both components are only at 10^{-10} M. By this and other criteria, the affinity of SC for all polymeric immunoglobulins is $> 10^{10}$ M^{-1}, sufficiently high for the complex to be essentially irreversible during its residency in the mucosal transport pathway.

MEMBRANE AND SECRETED FORMS OF THE RECEPTOR The effective irreversibility of IgA-SC interaction is important in explaining the cell biology of IgA transport. There are other examples of transcellular pathways for antibody; most notably, the transport of IgG across the placenta (98), and the import of IgG from the lumen of the neonatal intestine (99). IgA transport is unique in that a portion of the receptor remains with the ligand after exocytosis from the cell. Since IgA bound to SC is better able to resist

proteolytic cleavage (32), it has long been assumed that SC is sacrificed by the cell in order to protect its cargo against bacterial proteases. More likely, the cell has no choice in the matter: IgA is bound permanently to SC; it can only be released for secretion by jettisoning the receptor at the apical face. A frequent mechanism for ligand-receptor decoupling inside cells, exposure to a mildly acidic environment, is not available in this instance, since IgA-SC complexes are resistant to pH change (95). Thus, the protective effect of SC may be a fortuitous benefit accruing from other requirements of the transport pathway.

In biochemical terms, this generates a complication: SC must be secured in the membrane at one pole of the cell in order to capture IgA, but SC must be freely releasable at the other pole of the cell after transport. A conceivable solution is for SC to be bound first noncovalently to an additional protein in the membrane, from which it dissociates at a subsequent stage. This possibility was favored by Kuhn & Kraehenbuhl, when they detected SC-binding activity in mammary epithelium (100). Another solution is for SC to be synthesized initially as a larger peptide, with the lipophillic-anchoring extension cleaved from the molecule at the end of the transport process. When the molecular biology of the receptor was elucidated, it was the latter possibility that turned out to be correct.

A major accomplishment by Mostov et al (101) was the isolation of mRNA for SC from the rabbit mammary gland. When translated using a cell-free system supplemented with microsomal vesicles, a polypeptide was synthesized $\sim$20,000 M_r larger than SC isolated from milk ($\sim$70,000 M_r). This peptide traversed the vesicle bilayers, and $\sim$15,000 M_r could be removed from the C-terminus by digesting the intact vesicles with trypsin from what was equivalent to the cytoplasmic face. Demonstration that this form of SC was synthesized in vivo came with the isolation of the corresponding $\sim$95,000 M_r protein by affinity techniques from rabbit liver (102). The epitopes for soluble SC and several glycosylation sites are located in the N-terminal 70,000 M_r of the sequence.

Using rabbit tissue as the protein source was a fortunate choice, because IgA from the rabbit happens to bind SC by noncovalent interaction only (103). Rat IgA, like human IgA, forms a disulfide bond with SC (104). Probably for this reason, we have found the IgA transport receptor difficult to affinity-purify from rat liver. A crude receptor isolate contained multiple peptides at $\sim$100,000 M_r, but sufficient quantities have not been purified for proper characterization. However, when immune overlays are used on electrophoresed membrane preparations, 95,000–115,000 M_r proteins reactive with anti-SC have been identified in the golgi and plasma membrane of the rat hepatocyte (105). That the IgA receptor is initially synthesized as a transmembrane protein appears to be universal to all

species: mRNA for SC has also been isolated from the human carcinoma line and again produces a protein with a membrane-anchoring segment (46). There is probably only one expressed SC gene in man, implying that the secreted form of SC must derive from the larger transmembrane form.

CELL BIOLOGY OF SC SECRETION Biosynthetic labeling studies have elucidated the receptor's synthetic pathway (46, 47, 105, 106). First produced as a core glycosylated transmembrane protein in the rough endoplasmic reticulum, the secretory component acquires endo-H resistance via oligosaccharide modification in the golgi. It appears at the cell surface after ~30 min to pick up a molecule of IgA, and after a brief interval, migrates across the cell for secretion. Monoclonal antibodies prepared against the external and cytoplasmic portions of the receptor have been used elegantly to show that the entire peptide remains intact throughout these events until just before release from the cell, when the membrane anchoring C-terminal segment is removed (47).

Two questions linger: First, what is the protease that releases the secreted portion of the receptor from its membrane anchor? The primary sequence of SC from human whey reveals a ragged C-terminus (22). Perhaps this indicates that there is no dedicated IgA-receptor protease; rather, the receptor is released by hydrolysis at a labile site by any bystander protease that happens to be present in the organelles along the transport pathway. Second, what is the fate of the anchoring segment after cleavage? Since it cannot be reutilized, this segment is presumably degraded by the cell. But it is still unclear whether the anchor is separated away from the secreted portion in transit or whether it appears briefly on the apical membrane.

Endocytosis of the IgA Receptor—Is It Inducible or Constitutive?

Since the receptor is cleaved in the course of secretion, it obviously must be resynthesized for each trip across the cell. There are ~200,000 receptors on the surface of the hepatocyte (52) and presumably a comparable number on cells at other secretory sites. After IgA binds to the receptor, it is internalized within a very few minutes (56). Maintaining the full receptor complement at the plasma membrane therefore represents a very major metabolic commitment. One would suppose that evolution would have tended to minimize the expenditure by permitting endocytosis of the receptor only when it was occupied by a molecule of IgA.

Surprisingly, when Mullock et al (107) performed in vitro liver perfusions with an artificial medium, they found that SC was still being released into bile. Is there enough IgA resident in the donated liver to trigger the level of

SC secretion that is observed in these experiments? Over the course of a 5 hr perfusion with whole blood, we find that SC secretion is maintained fairly consistently, even after endogenous IgA in the perfusate is depleted. In fact, in the intact animal, most SC released into bile is in the free form, indicating that the system is less than 50% saturated by endogenous IgA. Apparently, the receptor shuttles across the cell and is secreted into bile, even without ligand triggering.

Why this transport system should be constitutive is not obvious. A simple explanation is that the valence of both ligand and receptor is one. Thus, the triggering signal typical for immunoglobulin receptors on mast cells and phagocytes—i.e. receptor cross-linking—is not a suitable mechanism to stimulate IgA uptake, and endocytosis must proceed continually without an inductive signal. Another possible explanation hearkens back to the cell biology of the system: Since SC is continually being synthesized de novo, perhaps it must be cleared regularly from the plasma membrane to prevent a transport backlog. Regulation of transport may only be possible at the level of receptor assembly, which may require several days to undergo significant change. Evidence for long-term modulation of SC transport has recently been obtained from mucosal biopsy specimens of IgA-deficient children (108). Within immunofluorescence staining, SC was still detectable in the intestinal epithelial cells. However, rather than being presented at the basolateral surface, SC had accumulated in the interior of the cell at the golgi.

Ligand Sorting and the Involvement of Other Receptors

Most vesicular transport pathways either initiate or terminate at points inside the cell. Transcellular pathways, of which IgA transport is an example, create problems of ligand sorting for the cells in which they occur. With the exception of IgA, protein endocytosed by the liver hepatocyte is largely either degraded in lysosomes or returned to blood. Asialoglycoprotein (ASG) is a model ligand for the degradative pathway (reviewed in 109). Even though IgA and ASG are processed within the same cell, there is $<5\%$ delivery of either ligand to the opposite destination (51). The two ligands are therefore sorted from each other—either on the cell surface, in lysosomes, or in an intracellular prelysosomal compartment (Figure 2).

It was easy to decide that the two pathways diverge at an early stage before the ligands reach lysosomes. Perhaps the most convincing evidence was that drugs that block lysosomal function and cause the accumulation of ASG in the liver do not obstruct IgA transport (51, 111). Distinguishing between cell-surface and early intracellular sorting has been more difficult. Certain drugs affect the two pathways independently (111), and ligands

from each pathway do not cross-inhibit (51). On the other hand, both ligands are initially endocytosed into vesicles of similar density (112). It is easily argued that these findings are circumstantial and do not address directly the issue of separateness of the pathways. The best morphological data is from Geuze et al (110). They showed that ASG and IgA receptors are sometimes compartmentalized together, and so they concluded that ligand sorting occurs intracellularly. This apparently conflicted with micrographic data published previously (113), which showed encapsulated ligands in vesicles that have different structural features.

Biochemical data on this question came from an unexpected finding. Human polymeric IgA1 (unlike IgA2, or IgA from other species) can bind to the ASG receptor (70, 114), probably through the unique *O*-linked oligosaccharides present in the hinge region. In the rat liver model, this means that human IgA1 is heterovalent—able to bind receptors for both the transcellular and degradative pathways (62). If prevented from binding

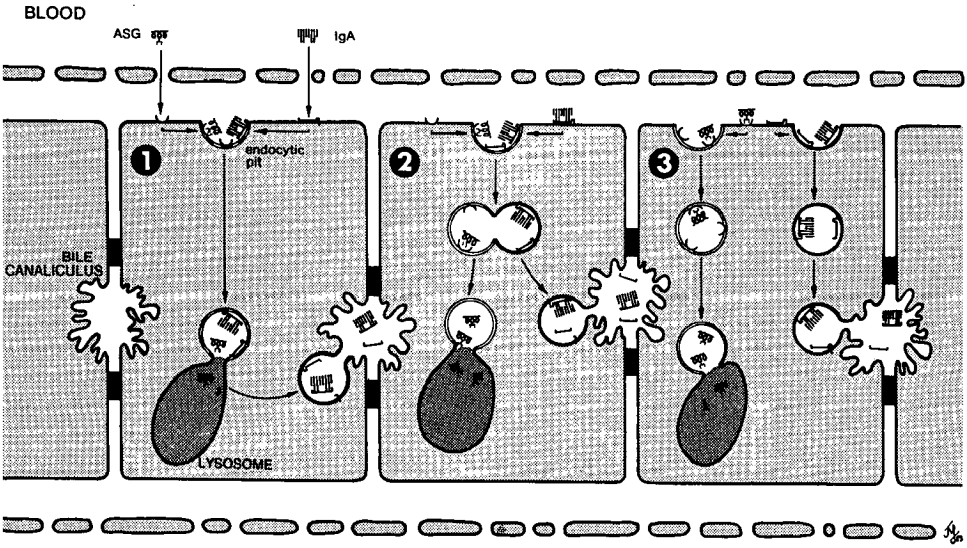


Figure 2 Alternative models for hepatocellular sorting between the mucosal transport pathway delivering IgA to bile and the degradative pathway delivering asialoglycoprotein (ASG) to lysosomes. The possibilities drawn show sorting at progressively earlier stages in the transport process. IgA never reaches the lysosome (model 1), but a ligand capable of binding both receptors can switch from one to another inside the cell (62). This could not occur if both pathways were completely separate (model 3). The data therefore support sorting intracellularly in a prelysosomal compartment (model 2). Immunoelectron microscopy has been used to identify a special tubular organelle near the sinusoidal membrane where IgA is peeled away from ligands intended for degradation (110). (Drawing reproduced from Ref. 51, by copyright permission of The Rockefeller University.)

to SC, human IgA1 is taken up by the liver for degradation. If prevented from binding to the ASG receptor, it is taken up and transported to bile, but at a rate significantly more slowly than for rat IgA. However, in the heterovalent state, human IgA1 is transported to bile just as fast as rat IgA; captured initially by the ASG receptor, human IgA1 switches to SC for rerouting to the bile canaliculus (62). The kinetic constraints imply that the receptor switching event occurs while transport is in progress—that is, while the two receptors temporarily occupy a common intracellular compartment before sorting (Figure 2, model 2). We expect that all published data can probably be consolidated, if the residency of SC in the common compartment is transient.

That human IgA1 can bind the ASG receptor creates a side issue which is nonetheless important. IgA normally survives in the circulation of humans for several days. In an overlay analysis, J. Baenziger has shown that polyclonal IgA1 is the one component of electrophoresed normal human serum with substantial binding activity for the isolated ASG receptor (personal communication). Primates, like rats, have an ASG uptake system capable of clearing small desialylated glycoproteins from portal blood on first pass through the liver (115). The enigma is therefore how human IgA1 manages to survive in the circulation of humans for so long.

Antibody Excretion Without SC: IgA-Producing Cells in the Liver

An important recent development related to IgA transport comes from Jackson et al (116). When immunized with a suitable antigen in Peyer's patches, rats soon begin to secrete IgA antibodies into bile. It is unlikely that this secretion occurs by an SC-mediated mechanism, because if the immunization strategy is changed, IgM or IgG antibodies appear in bile instead. Transfer experiments and in vitro liver perfusions have provided evidence that the specific IgA-producing cells migrate to the liver, where they may secrete their product directly into the biliary tract.

The impact of this work is multiple. First, it explains some outriding data on immunoglobulins in bile, attributed to the SC-mediated transport mechanism through hepatocytes but not easily reconcilable with our full understanding of that system. Second, these results account for the observed homing of labeled lymphocytes to the liver; such homing is often assumed to be simply an artifact due to damaged cells. Third, it suggests that the liver is an important contributor to secretory immunity by mechanisms distinct from SC-mediated transport. The phylogeny of this phenomenon is still unknown, but there is no reason to suspect that it will be as restricted as the SC-mediated hepatobiliary pathway. These findings might provide a new link between liver disease and the integrity of the mucosal immune system in man.

FUNCTION

Antigen Clearance from Blood

The physiological role of IgA in blood is poorly understood, especially in humans where it is found predominantly in the monomeric form, 5–10 fold more concentrated than in most animals (117). The only function of IgA that is reasonably substantiated by direct observation is antigen clearance. If circulating IgA originates partly from mucosal sites, then its immune specificity might make it especially useful for clearing the small quantities of dietary and microbial antigens absorbed from the gut into the systemic circulation (118). Inflammatory events accompanying infection can increase antigen absorption by the intestine, but IgA antibodies can attenuate this process (118). It may be important to clear dietary antigens quickly, to avoid stimulating a more widespread immune response that would divert the resources of the immune system away from the true cause of the infection.

Clearance of artificially created IgA-containing immune complexes has been studied by several groups. For reasons stated in a previous section, SC-mediated uptake of immune complexes probably plays a limited role under most natural circumstances, especially in species such as the mouse and man, where there is no substantial expression of SC on the hepatocyte surface. However, Rifai & Mannik (71) reported the uptake of IgA-containing immune complexes from the circulation of the mouse into nonparenchymal liver cells, especially Kupffer cells. This process is inhibitible only with other complexes bearing IgA; it is not mediated by SC, C3b receptors, or receptors for terminal fucose (119). Sancho and colleagues have reported the uptake of aggregated IgA by both parenchymal and nonparenchymal cells in the liver (120). There is some controversy as to how effective IgA antibodies really are in enhancing antigen clearance (121). Additional studies will be required to characterize the responsible receptors more fully and to evaluate their physiological importance.

Bienenstock & Befus (122) have proposed an immunogenic role for IgA, suggesting it may carry antigens in blood back to mucosal sites via SC-mediated transport. Outside the liver, the level of transport of IgA from blood to secretions is very low. Whether the small amount of antigen transferred in this fashion can play a role in sensitizing distant mucosal sites remains to be tested.

Immune Protection and Elimination

The prime biological function of IgA is obviously to protect the host from invading organisms that initiate their assault from outside the mucosal surface. Unfortunately, the effector mechanisms for IgA in secretions are difficult to document directly. Insight has been gained from a number of

experimental approaches, including: (*a*) in vitro experiments that evaluate the potential of IgA antibodies to affect microorganisms, either directly or in concert with effector elements, such as the complement system or cells, that mediate inflammation; (*b*) passive transfer experiments to determine whether IgA antibodies can protect a naive host against a challenge infection; (*c*) correlations between the level of specific IgA and elimination of infectious agents; and (*d*) studies on the consequence of selective IgA deficiency on disease susceptibility.

IN VITRO EXPERIMENTS: IMMOBILIZATION AND INHIBITION OF ADHERENCE The ability of IgA to bind antigen is in itself beneficial. Aggregation of microorganisms in the mucosal lumen would retard their movement to the mucosal surface and impair invasion of the host. The polymeric nature of secretory IgA provides not only increased overall avidity for antigen, but also an enhanced ability to cross-link multiple particles (123). The predominantly dimeric form of secretory IgA may reflect an evolutionary compromise between the cross-linking ability of IgM and the greater tissue permeability of monomeric immunoglobulins, such as IgG. It would be instructive to use exon shuffling techniques to assemble IgG, IgA, and IgM analogues with identical antigen-combining sites, with a view to examining the importance of these effects more closely.

When microorganisms such as virus particles succeed in reaching the epithelium, they attach to the membrane through receptors complementary to structures on the epithelial cell surface. Binding of antibody near these receptors on the organism's surface could prevent attachment by direct blocking, steric hindrance, or induction of a conformational change (124, 125). Similarly, purified secretory IgA antibodies have been shown to prevent the attachment of bacteria to musocal surfaces (126–128).

Under these circumstances, the ultimate effector of elimination may be nonspecific. Mucins that coat the mucosal wall provide a protective effect (129). Lysozyme, peroxidase, lactoferrin, and α-lactalbumin have important bactericidal and antifungal activities (1, 130). Cilia work actively to pass small particles out of mucosal areas such as the respiratory tract. By reducing the motility of microorganisms and preventing their adherence to the epithelial surface, IgA would render them susceptible to the natural cleansing functions of the mucosae.

Even if a pathogen succeeds in binding to the epithelium, IgA can still have a role to play. When neutralizing IgG is premixed with influenza virus, the virus is still able to enter target cells and be accumulated in the nucleus (131). When similar experiments are performed using specific secretory IgA (132), the virus again attaches to the cells but cannot be internalized. There is also evidence for a role as antitoxin: Specific IgA antibodies can minimize

the effect of cholera toxin (133), presumably by preventing attachment of its B subunit to host epithelial cells.

ANTIBODY-MEDIATED CYTOTOXICITY The ability of specific secretory IgA to participate in the direct killing of invading organisms is more controversial. Early work showed that *Vibrio cholerae* could not be opsonized by IgA (134). More recently, several investigators have identified receptors specific for the Fc region of IgA on peripheral and alveolar macrophages, lymphocytes, and neutrophils (135, 136). These Fcα receptors promote antibody-dependent cell-mediated cytotoxicity (ADCC) of IgA-sensitized target cells in vitro (136–138). IgA could therefore play an important physiological role in ADCC responses, providing that suitable effector cells are available in the mucosal environment. However, there have been several reports in which IgA inhibited the bactericidal activity of specific IgG (139–141). At sites accessible from blood, IgA could theoretically impair cytotoxicity by competing for antigen with IgG, but since specific IgG would predominate, the level of inhibition would be minimal. At the mucosa, less IgG is present, and there may be more Fcα receptor–bearing effector cells in the vicinity, so that IgA could assume an opsonizing role.

Aggregated IgA possesses a limited ability to activate complement by the alternative pathway (142, 143). This could add to the activation due to bacterial polysaccharides. However, IgA can also block classical pathway activation: Mouse monoclonal IgA antibodies inhibit the IgG-mediated lysis of red blood cells, either through direct competition for antigenic sites or through steric hindrance of the C1q binding sites of the IgG in the immune complex (144). This blocking effect may have pathologic consequences in bacterial meningitis, where meningococcal dissemination is associated with a lack of bactericidal activity in blood, and with the presence of specific serum IgA that can interfere with antibody-mediated lysis (139).

Pfaffenback et al (145) showed that mouse monoclonal IgA antibodies complexed with antigen could activate complement through the alternative pathway, but binding of C3b to the complex was weak, and the subsequent cascade of terminal complement components (C5–C9) was poor. The tendency of IgA and C3 to deposit together at selective tissue sites is strikingly illustrated in several clinical conditions, including IgA nephropathy, anaphylactoid purpura, and dermatitis herpetiformis (146, 147). The results of Pfaffenback et al (145) show how a primary deposition of IgA could lead to a chronic accumulation of C3, which in turn would have inflammatory sequelae. Whether the limited ability of IgA to activate complement plays a beneficial role in the mucosal interstitium is uncertain. In the lumen of the intestine, where complement components

are scarce, other biologic properties of IgA are undoubtedly more important.

PASSIVE TRANSFER STUDIES Designing a good transfer experiment to investigate the function of IgA is difficult. For example, because of the limited access from blood to the mucosa, intravenously injected IgA could not be expected to provide mucosal protection, except in the upper bowel of animals with an efficient hepatobiliary transport pathway. A more feasible approach is to provide secretory IgA to the lumen of a blind-ended ileal loop, segregated surgically from the rest of the intestine. Fubara & Freter (127) showed that secretory IgA, specific for *Vibrio cholera*, caused a five-fold reduction of bacterial attachment to the ileal wall.

Another approach is to preincubate a microorganism with specific antibody as a prelude to infectious challenge. Using this strategy, Lloyd & Soulsby (148) showed that specific IgA from colostrum or intestinal secretions could protect mice against an oral challenge with tapeworm oncospheres of *Taenia taeniaformis*. More recently, Kaplan et al (149) reported that specific IgA antibody against the intestinal protozoan parasite *Giardia muris*, when mixed with phagocytic cells, was able to render *Giardia* trophozoites noninfective. The ability of specific IgA to protect against challenge is generally equal to or greater than that of IgG on a mole-to-mole basis. Since IgA predominates in mucosal secretions, the bulk of antimicrobial antibody in the natural defense against these infections is undoubtedly of the IgA class.

CORRELATIVE STUDIES There have been a number of demonstrations that recovery from mucosal infection parallels the timing and magnitude of the secretory antibody response, better than it does the antibody response in blood. The literature is extensive, and it has been reviewed elsewhere (150–152). While these studies illustrate that local secretory immunity is subject to different regulatory mechanisms from the systemic immune system, they do not provide direct insight as to whether IgA participates in curing the host.

The preceding sections described reasonable mechanisms by which specific IgA in a previously immunized individual could confer protection against a fresh mucosal challenge. However, immune elimination is a different problem. Once an infection has taken hold, initial obstructions such as adherence have been partially overcome, and there is an enormous antigen load on the system. The value of an antibody whose primary strategies are direct neutralization and prevention of adherence might decline during infection, depending on the invasive strategy of the pathogen. If antibody must cooperate with other effector elements such as complement in order to assist in elimination, then the smaller amounts of

IgG and other immunoglobulins in the mucosa might be more useful. It is also possible that many infectious agents can be eliminated only by cellular mechanisms, provided by specialized intraepithelial lymphocytes (153, natural killers, macrophages, or mast cells. The correlation between recovery and increased secretory IgA levels in some clinical situations may just mean that humoral and cellular components of the mucosal immune system are regulated in parallel.

Comparison between susceptibility to disease and the ability to synthesize IgA is weaker, but this comparison is more informative in the present context. Inability to synthesize IgA is the most frequent immunoglobulin deficiency in humans, with a prevalence of 0.125–0.2% (154, 155). Increased prevalence in these individuals of a history of childhood infection (150), atopy (156), and circulating antibodies to food antigens (157) shows that the deficiency is not without its consequences. However, the majority of IgA-deficient individuals usually have no major health problems. Indeed, a compensatory increase in secretory IgM has been correlated with a lower prevalence of infection in IgA deficient children (158). This compensation might occur because the same immunocompetent cells are still capable of homing to the mucosa but are unable to undergo class switch from IgM to IgA, due to the genetic defect. The effectiveness of this substitution suggests there is no effector function exclusive to IgA that is an absolute requirement for health.

EPILOGUE

The secretory immune system has evolved with specialized features that reflect the priorities required for frontline defense in the mucosa. There are regulatory mechanisms, antigen presentation systems, and cell types that are distinct from their counterparts in the systemic immune system. As the primary mode of humoral immunity in secretions, IgA has evolved not to trigger a large repertory of Fc-mediated effector mechanisms (since many of these are not available in secretions), but to resist proteolytic attack of invading microorganisms and effectively to prevent them from gaining a foothold on the mucosal wall. Subsequently, most potential pathogens are disarmed by the nonspecific factors present in secretions and pass harmlessly out of the system. IgA may also limit the systemic immune response during times of health by clearing antigens from blood. Less certain is whether IgA plays a role in limiting the course of a major infection already underway.

The SC-mediated mechanism delivering IgA into secretions is the most active and widespread transcellular protein transport system in the body. Just to maintain the continual shuttle of IgA receptors across the cell

requires the synthesis of roughly 30 μg SC protein hr^{-1} per g tissue. Investigating this phenomenon has helped address some fundamental questions in cell biology, relating to intracellular traffic and ligand sorting. By genetic recombination of elements from different protein processing pathways, it will soon be possible to define the factors that regulate these events.

Even though the effector mechanisms of IgA may be unspectacular, the expenditure undertaken to provide IgA to mucosal sites underlines its importance. The essential nature of humoral immunity in secretions is further illustrated by the redundancies built into the system: When IgA synthesis is deficient, IgM-producing cells localize in the mucosa instead. And, not to rely completely on SC-mediated transport, immunoglobulin-producing cells home to the liver and secrete directly into bile, thereby providing an additional source of antibody for the intestine. Just recently, the wasted mouse was described as a disease-susceptible strain that is depleted of any humoral secretory immune system (159). Perhaps because of the multiple redundancies present, such a generalized secretory immune deficiency is rarely detected in humans. The mucosal IgA system is an important strategic defense initiative that is designed and regulated to obtain maximum benefit from the required metabolic investment.

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Due to space limitations, the bibliography that follows is only a restricted sampling of the extensive recent literature on the immunobiology of IgA. Omission of a particular article from this list should not be taken to reflect adversely on the quality or importance of the work. To the many investigators who have not been cited, but who have contributed to the development of ideas presented in this review, our apologies and thanks.

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CONTENTS

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THE RECEPTOR WITH HIGH AFFINITY FOR IMMUNOGLOBULIN E[1,2]

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INTRODUCTION

This review is about one of the physiologically important macromolecules that interact with the Fc domains of immunoglobulins. Such substances (complement, "immunoglobulin binding factors," Fc receptors) can convert the simple binding of antigen to antibody into a biologically meaningful event.

The protein we review here—the Fc receptor with high affinity for monomeric immunoglobulin E (IgE)—is only one of several proteins that interact with IgE (1–5). In addition to high affinity, its special characteristic is its distribution on the plasma membrane of mast cells and basophils exclusively. Activation of this receptor initiates release of preformed mediators contained in intracellular granules and the biosynthesis of a variety of biologically active lipids such as the leukotrienes. Here we review what is known about the structure of this receptor and the early biochemical events that it initiates. We do not consider the consequences of

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[2] Abbreviations: IgE, immunoglobulin E; RBL, rat basophilic leukemia; PI, phosphatidyl inositol; PIP, phosphatidyl inositol-4-phosphate; PIP_2, phosphatidyl-4,5-bis phosphate; AdoMet, s-adenosylmethionine; AdoHcy, s-adenosylhomocysteine.

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these initial perturbations—an area that deserves separate discussion. Because our subject has been regularly reviewed (6–8), we focus principally on recent data.

MATERIALS AND METHODS

IgE and Cells

Molecular characterization of receptors requires reasonable amounts of material. With respect to the ligand, human IgE myelomas have been available since the late 1960s (9), but the lack of suitable preparations of human cells was a serious drawback. Modest numbers of mast cells can be purified from rats and mice, but preparing sufficient rodent IgE was laborious (10). The discovery by Bazin and his colleagues of rat immunocytomas (11), many of which secreted homogeneous IgE (12), was a major breakthrough. The development of the monoclonal antibody technology (13) now permits the preparation of virtually unlimited numbers and amounts of antigen-specific mouse IgEs. One of these—HI-DNP-ε-26.82, which is specific for the dinitrophenyl moiety (14)—has been particularly popular.

The unique development of a *basophilic leukemia* in rats treated with β-chlorethylamine was first reported in 1973 (15). In vitro culture of these RBL cells was initiated in our laboratory that same year (16) and independently by Froese and his colleagues in 1975 (17). These initial isolates are apparently the source of all subsequent work on these widely used cells. By several criteria these cells seem most closely related to so-called mucosal mast cells (18).

The initial cell lines appeared granular and had low but palpable amounts of histamine and substantial numbers of receptors; even so, they failed to undergo receptor-mediated secretion (16). Subsequently secreting cell lines arose spontaneously—an event so unexpected that the cells were originally thought to have arisen from a mouse mastocytoma (19, 20). Cloning of the releasing cells led to a line, 2H3(HR+), (21) which has continued to be highly functional. Recently, other cell lines have been prepared that will be useful for comparative purposes (22).

The IgE-mediated release from RBL cells has many of the characteristics of release from human basophils and rodent mast cells (23). Similarly, no substantial discrepancy between the structure of the receptor on the RBL cells and that on normal cells has been uncovered, although only limited comparative data are available (17, 24–27). The differences that do exist, rather than diminishing the usefulness of the tumor cells, may assist in sorting out the essential from the trivial. Likely, this will be equally true for the reactions initiated by the receptor.

Purification of the Receptor

Once solubilized, the receptor can be absorbed to IgE-adsorbents. After washing, the receptor can then be released by use of a variety of protein-denaturants (7, 28, 29). There are problems with this approach. (*a*) Since the adsorbents will likely contain aggregates of IgE as well as monomeric IgE, cellular proteins with low affinity for monomeric IgE may coabsorb (1). (*b*) The aggregated IgE may activate those receptors absorbed to it—if such an event as "activation" indeed occurs (see below). (*c*) The use of denaturants can dissociate the receptor (30) and may induce functional changes. Since no functional assays as yet exist, such changes are undetectable. (*d*) Nonspecificically absorbed contaminants may also be released by the denaturant.

An alternative approach involves binding monomeric IgE to the receptor either before or after solubilization and then trapping the IgE either on an antihapten adsorbent utilizing hapten-conjugated IgE (31, 32) or on a hapten-conjugated adsorbent using antigen-specific IgE (33). After washing, the IgE-receptor complexes can be eluted with hapten without employing denaturants. The slow rate of dissociation (34) prevents appreciable losses of receptor throughout purification especially when nmolar concentrations of IgE and receptor are used. The disadvantage of this approach is that IgE-receptor complexes, rather than unliganded receptors, are purified. However, the binding of monomeric IgE to the receptor is not known to lead to any changes in the receptor (see below), so that in principle this is not a serious problem. With this procedure 30% yields of intact receptors can be isolated. Most current lines of RBL cells have approximately 3×10^5 receptors per cell and can be readily grown to a density of 10^6 cells/ml. Thus 10 liter of cells can yield ~ 1.5 nmol receptor.

STRUCTURE OF THE RECEPTOR

Analysis of the receptor was initiated in the mid-1970s in several laboratories, and progress has been regularly reviewed (6, 7). In 1983 we presented a new model for the receptor (35), and we use that model as the basis for discussing recent observations. We focus on several major aspects: The properties of the subunits that constitute the unit receptor; the topology of these subunits in the membrane; the interaction of the receptor with detergents and lipids; and some questions of definition.

Covalent Structure

PROTEIN CHEMICAL APPROACHES The model of the receptor contains four polypeptide chains: a single α chain (36), which contains the binding site for

IgE (17, 24), a single β chain (26, 33); and two identical, disulfide-linked γ chains (27) (Figure 1). Some of the properties of these chains are collected in Table 1.

New findings support specific features of this model. On the basis of the products of proteolytic cleavage of the α chain (46) and, later, on the basis of the kinetics of proteolytic cleavage of receptor bound IgE (47), we proposed that the α chain consists of two similar-sized but nonidentical domains. That the Fab fragments of a particular monoclonal antibody directed to the α chain (antibody BC4) bind in a ratio of $\sim$2 mols per mol receptor (48) is consistent with this proposal. We also proposed that the two γ chains are identical, and two-dimensional analysis of intact γ chains subsequently confirmed that conclusion (unpublished observations).

That the γ components are not degradation products of either α or β has been further confirmed by two-dimensional chymotryptic peptide maps of extrinsically iodinated α, β, and γ, which show discrete patterns with essentially no common peptides (G. Alcaraz, J.-P. Kinet, H. Metzger, unpublished observations). This confirms the distinctiveness of the γ chains and implies that lengthy runs of sequence identity between them and β or α are unlikely. We have insufficient data to show that by the same criteria this is also true for the relationship between α and β.

GENETIC APPROACHES To explore the sequences and sequence-relationships further, cloning of the genes that code for them is the most reasonable strategy. We plan to prepare cDNA probes synthesized on the basis of limited sequence analysis. Because the amino terminus of each of the chains appears to be blocked (36; J.-P. Kinet, G. Alcaraz, H. Metzger, unpublished observations, in collaboration with M. Bond), isolation of one

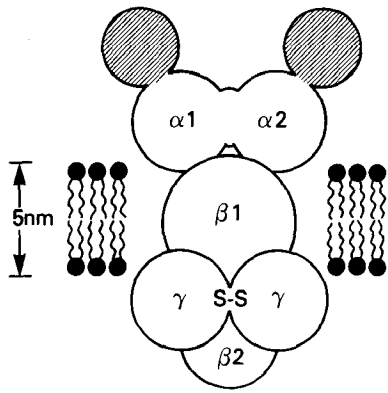

Figure 1 Schematic diagram of receptor for immunoglobulin E. The circles represent spheres whose volumes are proportional to the mass of the peptide and carbohydrate (shaded areas) they define. From Ref. 35 by permission.

or more peptides is necessary to obtain such data; isolation of tryptic peptides has been initiated.

An alternative approach is to prepare cDNA from mRNA with or without prior fractionation of the latter. Using RBL cells, Pure et al (49) as well as Liu & Orida (50) obtained 13-14S fractions of mRNA from sucrose gradients that, when injected into frog oocytes, induced binding activity for IgE. Analysis of this IgE-binding material on polyacrylamide gels showed principally a 31 kd component of uncertain relationship to either the α, β, or γ chains (50). Liu et al (51) have cloned the double-stranded cDNA prepared

Table 1 Properties of the subunits of the receptor for immunoglobulin E[a]

Properties	Subunit		
	α	β	γ
Mol/mol receptor (35)[b]	1	1	2
M_r (kd) (27, 33, 37)	45	33	9
Labeling properties			
Extrinsic			
Surface			
Intact cells, vesicles (17, 24, 38)	+	–	–
Lysed vesicles (38)[c]	–	+	+
Intramembrane (27, 39)	–	+	+
Intrinsic			
Glucosamine (24, 27, 40)	+	–	–
Phosphorus (41, 42)[d]	–	+	+
Fatty acids (45)	+	+	+
Composition			
Carbohydrate (wt%) (36)	30%	–	–
Amino-acids (mol%) (36)[e]			
Basic (Lys, Arg, His)	14.6	11.2	15.7
Acidic (Glx, Asx)	20.7	20.9	20.7
Hydroxy (Ser, Thr)	16.1	19.4	12.3
Hydrophobic (Val, Leu, Ile)	22.7	23.6	26.1
Aromatic (Tyr, Phe, Trp)	11.3	8.3	8.0
Other			
Cys	2.36	2.05	1.92
Met	1.04	1.3	0
Gly, Ala, Pro	14.0	18.6	17.1

[a] Data from RBL cells exclusively.
[b] Reference.
[c] Refers to labeling in addition to that seen on intact vesicles.
[d] Refers to intact cells only. Hempstead et al (43, 44) report labeling of α chains on rat peritoneal mast cells.
[e] The data for the β and γ chains are from G. Alcaraz, T. Y. Liu, J.-P. Kinet, H. Metzger (unpublished studies).

from such mRNA fractions, and this cDNA in turn hybridizes with the mRNA coding for the 31 kd protein. In the amino acid sequence published so far (encompassing approximately one half of the peptide), there appear to be five methionines—more than our own analysis suggests for either the α, β, or γ chains (Table 1). Liu et al suggest that possibly their protein represents the similar-sized material described by Kulczycki & Parker (29) and Holowka & Baird (38).

The relationship of the protein described by Liu and his colleagues to the α, β, and γ chains could be clarified with suitable antibodies. Reports exist of anti-α-chain antibodies (7, 48, 52, 53), but no rigorous analysis of their specificity or of their cross-reactivity with the cloned protein has been published. We (and perhaps others) have tried to prepare antibodies specific for β and γ but as yet we have not succeeded.

Topology

New evidence supports one or another aspect of the topology of the receptor, as depicted in Figure 1. Holowka & Baird (38) used lactoperoxidase to label regions of the receptor exposed on plasma membrane vesicles prepared by treating RBL cells with formaldehyde and dithiothreitol (54). The intact vesicles, which appeared to be primarily right side out, revealed labeling of the α chain almost exclusively. Labeling after lysis of these vesicles produced no increase in the modification of α but now revealed labeling of β and γ. These results are consistent with the model. The results of Lee & Conrad (55) are also confirmatory. As we did also (26, 56, 57), they observed covalent cross-linking of α, β, and γ when bifunctional reagents were reacted with the solubilized receptors. However, when membrane-impermeable cross-linking reagents were reacted with intact cells, no cross-linking was evident (55). [With membrane-permeant reagents such as the bis-imidates, cross-linking of α, β, and γ on intact cells is obtained (26).] These observations are consistent with minimal or no exposure of the β and γ chains on the surface (Figure 1). Although some additional analyses are needed to improve the accuracy of our amino acid analyses, the current data (Table 1) show that the general distribution of hydrophobic and hydrophilic amino acids in α, β, and γ is similar. Though enriched in hydrophobic residues compared to soluble proteins in general (58), each chain has numerous hydrophilic and charged residues; this indicates that each is partially exposed to an aqueous environment.

We emphasize that the current topological scheme is based in part on negative data and in part on no data whatsoever. For example, there are no data that allow one to speculate whether α interacts with β or with γ. Only a hint exists that α does not independently interact with both (see below).

Interaction with Detergents and Lipids

DETERGENTS An unusual feature of the $\alpha\beta\gamma_2$ complex is its dissociation in so-called mild detergents (30, 56), and it was this property that delayed detection of the β and γ chains. Two observations have allowed us to examine this dissociation in detail. The first was that the instability could be largely obviated by maintaining the solubilized receptor in submicellar detergent (30, 59). The second was that by briefly exposing the receptor to micellar detergent in the absence of lipid and then maintaining it in submicellular detergent, even the α chain in the IgE-$\alpha\beta\gamma_2$ complex could be labeled (see also below) (30, 45, 59).

Our principal findings on the dissociation of β and γ_2 from α were as follows (30): (*a*) The β chain and dimer of γ chains always dissociated in unison. This is a hint that β and γ don't interact independently with α (Figure 1). (*b*) The dissociation is due to detergent micelles and, under appropriate conditions, the extent of dissociation correlates well with the concentration of micells regardless of which of six different commonly used mild detergents is employed. (*c*) Under otherwise mild conditions, the molar ratio of detergent monomers (in micellar form) to receptor that is required for dissociation is about 10^5. This ratio is strikingly higher than the ratio of detergent to lipid that is required to dissolve membranes, where a ratio of $\sim$2 suffices (60, 61). (*d*) Detergents that are most effective for dissolving biomembranes—those with an HLB number (62) of about 13 (63)—disrupt the receptor most efficiently. (*e*) At low levels of micellar detergents, the kinetics of dissociation implicate a component other than the receptor and the detergent, that influences the process. We believe this component is lipid that is tightly associated with the receptor (below).

The dissociability in mild detergents of the β and γ chains from the α chains is useful for preparatory isolation of the subunits, especially when Triton X-114 is employed (59, 64). With this detergent we can separate the α subunits of the receptor from the β and γ chains under conditions that maintain high concentrations of all the subunits and that are otherwise extremely gentle (59). Nevertheless, we have not so far been successful in recombining the β and γ chains with α, once they have been dissociated.

LIPIDS Recent studies indicate that lipids bind to the receptor in several ways (45). Evidence for *loosely* bound lipid is derived from observations on the conditions necessary to iodinate extrinsically the α chain of the receptor in the IgE-$\alpha\beta\gamma_2$ complex (see above). Previous data had indicated that α could not be oxidatively iodinated once bound to IgE (17, 25). We found that if the complex was briefly exposed to lipid-free micellar detergent and then iodinated in submicellar concentrations of detergent, substantial

iodination of the α chain is obtained. If micellar detergent, with or without lipids, is added back to the complex prior to iodination, the labeling of α is again suppressed. These observations suggest that there are sites on or near the α chain to which lipid binds weakly and that these are rather nonspecific.

Evidence for *tightly* bound lipid comes from the observation that phospholipids prevent the disruptive action of detergent (45, 56), and from the dissociation studies cited above that implicated a third component. Cell-derived mixed lipids are the only ones that have so far been found to protect fully against the disruption induced by detergent (45, 56); this suggests that the tightly-bound lipid may be rather specific. A direct analysis to test this proposal has been initiated (65).

The receptor also contains *covalently* bound lipid (45). Because only small amounts of radioactive fatty acids are incorporated, it is impractical to assess the molar ratio of fatty acid to polypeptide. In the absence of knowledge of whether a substantial proportion of the receptors are modified, it is futile to speculate about the potential function of such lipid.

Defining the Receptor

β and γ_2 dissociate from α under conditions in which most multisubunit membrane proteins remain intact, and the β and γ chains associated with α chains already inserted in the plasma membrane incorporate radioactive phosphorus (42) and fatty acids (45). These facts raise the question of whether it is appropriate, therefore, to consider the β and γ chains as true subunits of the receptor rather than as receptor-associated components. Once the molar ratio has been defined as reasonable (a small integral number), we consider the most decisive criteria to be biosynthetic ones. An examination of the biosynthesis more detailed than had been previously performed (27) has been published (66). These new experiments showed that α, β, and γ are coordinately synthesized and degraded, and cannot exchange independently of each other once inserted into the plasma membrane. It, therefore, seems reasonable to consider the $\alpha\beta\gamma_2$ complex as the unit receptor.

BINDING OF IgE TO THE RECEPTOR

Reaction Mechanism, Kinetics

The principal findings have been reviewed (e.g. 67) and can be summarized easily. All the evidence favors a simple bimolecular, nondiffusion-controlled interaction between a monomer of IgE and a single univalent receptor to which it binds (34, 68, 69). The forward reaction can be described by a single-valued rate constant of about 10^5 M^{-1} s^{-1} for intact cells and

about 30-fold greater for solubilized receptors. The reason for this discrepancy is uncertain. Possibly the slower rate is due to a "gating" effect of other glycoproteins on the cell surface (69). Alternatively, the bending of IgE, which may occur when it binds to cell-bound receptors (see below), may not happen when IgE binds to solubilized receptors (70). The former proposal can be studied by using receptors reincorporated into liposomes free of other proteins (57); the latter, with comparative studies using energy transfer techniques (70, 71). The forward reaction is unremarkable either in its rate constant or in its sensitivity to pH, temperature, salts, etc (34, 72). The dissociation is slow and has a concentration dependence appropriate for a unimolecular reaction. When studied over long time periods, the kinetics suggest that the receptors are heterogeneous since the dissociation data show a concave-upward shape on a semilog plot (73). The shape of this plot is not influenced by the fractional occupancy of the receptors (69), so there is no reason to invoke any (negative) cooperative effect. These data likewise preclude a diffusion-controlled reaction (69). Thus, the slow rate of dissociation ($k_{-1} < 10^{-5}\ s^{-1}$) accounts for the high affinity of the receptor for IgE.

Specificity

The specificity for the epsilon isotype is virtually perfect—no homologous monomeric isotype can compete effectively against the binding of IgE. The species specificity is imperfect. For example, rodent IgE can bind to human cells (74), and recent evidence was presented that human IgE could bind to rodent cells (75). The former observation is not surprising; it is only surprising that it had not been adequately tested previously. The latter finding is unexpected because it had earlier been found that human IgE cannot block the binding of rodent IgE (16, 76). In those instances the human IgE may have become largely inactivated, but because very large concentrations of the IgE were used, this explanation seems improbable. Another unusual aspect of these recent findings is that RBL cells failed to bind human IgE, whereas peritoneal mast cells did (75). Previous studies had suggested very similar binding properties for these cells (76, 77), and peptide maps failed to pick up reproducible differences in the α subunit which binds IgE (25). Some older studies had suggested that human reaginic sera could sensitize rodent mast cells (reviewed in 67); this could have resulted from minimal binding of IgE or perhaps from binding to other receptors. It was in any case difficult to reproduce (78).

Binding Sites on IgE

The binding of IgE to the receptor is not known to cause any significant perturbations by itself. Therefore, definition of the sites on IgE that interact

with the receptor is unlikely to clarify the mechanism of action of the receptor. The interest in this area of research is prompted, rather, by the therapeutic implications of success. That is, if one could prepare suitable peptides based on knowledge of the interactive sites, one might have a useful way of preventing sensitization of mast cells and basophils with endogenous IgE.

OLDER DATA A problem in this line of investigation has been the failure to obtain small fragments of IgE that are active. Human IgE can be split by proteases to produce active Fc fragments [$(C_\varepsilon2\text{-}C_\varepsilon3\text{-}C_\varepsilon4)_2$] (79–81), but no smaller fragment has been found to be reproducibly active (reviewed in 47).

Dorrington & Bennich (82) correlated the inactivation of binding of human IgE with the irreversible changes induced by denaturants and concluded that both the $C_\varepsilon3$ and $C_\varepsilon4$ domains—particularly the former—were involved.

Perez-Montfort & Metzger (47) attempted to generate active fragments by carefully controlled proteolytic digestion of rat and mouse IgE in the presence and absence of receptor. Although no active fragments (not even an Fc fragment) could be generated, the kinetics of cleavage at several sites yielded some useful information. The presence of receptor slowed (but did not totally inhibit) digestion at sites presumed to be between $C_\varepsilon3:C_\varepsilon4$ and $C_\varepsilon3:C_\varepsilon2$ (mouse IgE) or within $C_\varepsilon3$ and between $C_\varepsilon3:C_\varepsilon2$ (rat IgE). The latter cleavage was most profoundly affected, the rate being sufficiently slow that the breakdown might actually have been of the small amount of IgE that had dissociated during the course of the experiment. These results clearly implicate a region close to the $C_\varepsilon2:C_\varepsilon3$ juncture on each epsilon chain as one that interacts with the receptor.

NEW RESULTS

Genetic Approaches Two groups have infected bacteria with plasmids bearing an insert of the cDNA that codes for all or most of the Fc region or the human epsilon chain (83, 84). The bacteria produced unglycosylated Fc fragments that bound to human mast cells or basophils with the requisite specificity. These results confirm previous data (with rodent IgE) that carbohydrate does not importantly contribute to the binding of IgE (85). The fragment isolated by Liu et al (84) is missing the NH_2-terminal one third of the $C_\varepsilon2$ region, so this region is apparently also not required.

Both Kenten et al and Liu et al had to use rather harsh conditions to isolate and purify the epsilon fragment dimers. This is not a trivial matter for the following reason. Both groups indicate that it will now be possible to perform site-specific mutations and to study their effect on the binding to the receptor. Such mutations may, however, change the sensitivity to, or reversibility of, the denaturing conditions used to prepare the material. It

will be difficult to disentangle losses in activity due to such effects from those due to alterations at the binding sites themselves.

Even if conditions could be found whereby the use of denaturants could be avoided, the underlying assumption of all such studies, i.e. that an exchange of a specific amino acid will have only local effects, is not universally true (86). It is likely that many mutants will have to be screened before an interpretable pattern emerges.

Physicochemical approaches Holowka & Baird and their colleagues have employed energy transfer techniques to assess the interaction of IgE with receptors on cells or on membrane vesicles (54, 70, 71, 87). They inserted acceptor probes into the bilayer via hydrophobic tails and measured the quenching of fluorescent donor probes chemically attached to various sites on IgE, or on antibodies to IgE, and of a fluorescent hapten in the combining sites. Energy transfer between probes on antibodies that bind at or near $C_\varepsilon 1$ and somewhere in $C_\varepsilon 3$ or $C_\varepsilon 4$ (88) was also measured. The quenching data can, with certain assumptions, be interpreted to yield an average distance between the donor and acceptor with an estimated accuracy of $\pm 20\%$.

Such studies yield only indirect information and require too lengthy an interpretive analysis to review here. The authors conclude that the IgE binds via only one face of the Fc region and bends at the $C_\varepsilon 2 : C_\varepsilon 3$ interface. Bending at the analogous interface in IgM has been proposed, and this is also the principal locus of the limited segmental flexibility of IgM and IgE. It is, therefore, surprising that no substantial changes in the flexibility of IgE occur when it binds to the cell-bound receptor (89). More direct alternative approaches will be necessary to confirm the model of Holowka et al. It is, however, consistent with the observation that interactions occur well removed from the distal end of the Fc and that the terminal portions are not totally prevented from interacting with enzymes (47) or antibodies (88).

Studies with monoclonal antibodies Another way to identify the regions on IgE critical for binding to the receptor is to employ monoclonal antibodies. Baniyash and coworkers (90, 91) have described a monoclonal antibody "51.3," which binds to the Fc portion of mouse IgE and has some interesting properties. The antibody blocks the binding of IgE to RBL cells but only partially—55–65% (90). Nevertheless, this antibody reacts with cell-bound IgE, albeit to a lesser extent than to unbound IgE. When Fab fragments of the anti-IgE were prepared and the stoichiometry of their binding assessed, Baniyash et al (91) estimated that 3 mols Fab bound per mol IgE in solution and only 2 mols per mol with cell-bound IgE. These observations could be related. We suggest there may be two pairs of similar epitopes that this antibody recognizes. If the antibody binds to sites of one set (A) first, it

inhibits binding of antibody to the other set (B) but does not inhibit binding of IgE to the cell. Contrariwise, those molecules of IgE to which antibody binds at site B first are inhibited from binding to the receptor and have a reduced capacity to interact with the antibodies at site A. This could explain the partial inhibition of binding, a phenomenon based on a stochastic event, rather than on the heterogencity of receptors, as the authors suggest (90). Our proposal could also explain the decreased binding of the antibodies to cell-bound IgE. The B sites (above) are unavailable on cell-bound IgE, whereas the A sites would still react with antibody.

Differences in the reactivity of free and cell-bound IgE and the apparent discrepancy in the observation that antibody can inhibit binding but still react with cell-bound IgE, could also be explained by changes in the conformation of IgE when it binds, as noted above.

Other strategies Each of the approaches described so far yields results that are difficult to interpret unambiguously. It is appropriate therefore to consider other stratagems. One is to compare the sequences of IgE from different species with each other and with sequences of other isotypes of immunoglobulin. Given the partial cross-reactivity of mouse and human IgE with the corresponding heterologous receptors (above), it is difficult to know what to look for! Notably, the least divergent portion of the mouse and human epsilon chain is in $C_\varepsilon 4$ (92)—a region that is implicated to a lesser extent than $C_\varepsilon 3$ (above). It seems likely that comparative sequence analysis can at best provide a basis for deciding on which site-specific mutations it would be most profitable to focus (see above). We previously (6) suggested alternative approaches that still seem worth exploring by those interested in this area: (*a*) Use of cross-linking reagents and (*b*) modification of IgE in the presence and absence of the receptor. Although the work in analyzing the appropriate peptides will be tedious, the results are likely to be less ambiguous than those produced by most other methods.

AGGREGATION AS THE INITIATING SIGNAL

We shall not recount once more the oft-reviewed extensive evidence that aggregation of the receptors is the initiating signal in IgE-mediated release (6). Rather, we shall focus on newer studies that relate to several specific aspects of such aggregates.

Size of the Aggregates

Earlier studies had suggested that aggregates of IgE as small as a dimer might be sufficient to initiate release (93, 94) but the possibility that minor quantities of larger aggregates were in fact the critical stimuli could not be

excluded. The quantitative analysis by Segal et al (95) appeared to settle the issue, since this showed that highly purified, covalently cross-linked dimers could indeed initiate release from rodent mast cells. Similarly, human basophils were shown to be stimulated by dimeric IgE (96). The water was only a little muddied when Fewtrell & Metzger (97) observed that with RBL cells, dimers were virtually inactive in the absence of D_2O (below), and even in its presence were considerably less effective than trimers, and the latter less than higher oligomers. Analogous studies have been performed by Menon et al (98) using a monoclonal anti-IgE (A2(88)) that at low concentrations forms dimers of IgE almost exclusively but which at higher concentrations can form larger aggregates (71). With this reagent, peritoneal mast cells show the same poor response to dimers as is shown by RBL cells, whereas with preformed dimers of IgE, Segal et al studying cutaneous mast cells (95) and Fewtrell & Metzger using peritoneal mast cells (97) found excellent responses. Because putatively similar cells may show substantial differences in their responsiveness to dimers (below), experiments with both types of reagents need to be performed with a single batch of cells in order to determine the basis of this variability.

The initial studies on human basophils yielded quantitatively less dramatic differences between dimers, trimers, and higher oligomers but the results were qualitatively similar to those reported on RBL cells (96). More recent studies by MacGlashan & Lichtenstein show a correlation between the "releasability" of cells and their ability to discriminate between dimers and higher oligomers, i.e. their ability to respond to dimers (99, 100). It is known that the peripheral blood basophils of 10–20% of donors fail to release when challenged with antigen or anti-IgE. Other donors respond to different degrees even when care is taken to compare cells that have equivalent quantities of bound IgE. MacGlashan & Lichtenstein find that cells that are more responsive in general also respond well to dimers, whereas those that respond sluggishly in general respond poorly to dimers but quite adequately to trimers (100).

A fundamental question about the interpretability of the studies with small oligomers of IgE is raised by a recent observation of Holowka & Baird's group. They showed that cell-bound small oligomers of IgE with a multiplicity <6 slowly coalesce and form larger clusters containing 100s to 1000s of molecules (101). This raises the question of whether the concept of a "unit signal" being as small as a dimer of receptors must now be discarded. The implicit assumption of the original proposal (95) was that oligomers of IgE whose *maximum* size was well defined would induce aggregates of receptors, the multiplicity of which had a similarly well-defined upper limit. If this is not the case, then although it might still be true that a dimer could initiate the chain of events, the size of the mechanistically active aggregate

of receptors would remain uncertain. Holowka & Baird and their colleagues have provided data that suggest the original proposal is still likely to be correct. Allowing receptors occupied by small oligomers to coalesce into larger aggregates prior to initiating release by a temperature jump had no effect on either the magnitude or the kinetics of the subsequent degranulation (98). Thus, although it is difficult to rule it out completely, the coalescence of aggregated receptors into larger clusters does not seem to play an essential role, and with responsive cells, dimers of receptors appear to be capable of generating at least a modest or, in some cases, an optimal signal. Whether this signal is adequate to induce substantial release appears to be related to other factors, still undefined. Where the question has been examined, all the perturbations that appear to reflect early events (see below) exhibit the difference between stimulation by dimers and trimers, etc.

Number of Aggregates

Localized aggregation of receptors by reagents bound to beads induces only localized degranulation (102). Furthermore, all the quantitative analyses suggest that more than a single aggregate of receptors is necessary to get maximum degranulation. But these studies also indicate that only a few per cent of the total receptors need to be aggregated to observe substantial release (23, 97). Nevertheless, the dose response curves are relatively shallow, and per unit of release, progressively larger numbers of receptors must be engaged as maximal release is approached (97). There are insufficient data to assess the stage at which the relative refractoriness occurs, but it is likely to be an early one. For example, the breakdown of phosphatidyl inositols (PIs) can be studied on RBL cells (see below) using trimers of IgE, and the maximum number of receptors that could have been aggregated can be determined from the number of trimers that were bound. Detectable breakdown is observed when no more than ~ 100 receptors per cell could have been aggregated (103), but even though more and more receptors become aggregated, a proportional increase in breakdown of PI does not occur. It is likely that the progressive disproportionality is amplified by the process of desensitization (see below). In any case, until some of the fundamental biochemical phenomena in both activation and inactivation can be identified positively and studied independently, the relationship between the numbers of receptors aggregated and the amount of release probably cannot be studied rigorously. On the other hand, it is already clear that the maximum release of mediators attained is not limited by either the number of receptors aggregated nor, for example, by the amount of PI broken down. Thus, maximal (but incomplete) release is

obtained before either of the latter two phenomena have reached a maximum (103).

Mechanistic Role of Aggregates

In later sections, we review a variety of phenomena each of which has been reported as an early consequence of aggregation of the receptors. The role of the receptor has not been defined for any of these events. In this section we, therefore, consider some general aspects of this problem and review what progress has been made in its solution.

INTRINSIC ACTIVITIES We previously indicated that it is useful to consider two classes of possible mechanisms (6). Upon aggregation, the receptor itself may assume one or more functions, such as forming a channel for ions or carrying out an enzymatic activity. The first steps in testing for such *intrinsic* activities have been accomplished: Methods of purification have been devised that obviate the need for using ordinary denaturants (32), and the deleterious effects of the mild detergents necessary to solubilize the receptors can be minimized either by maintaining a low micellar detergent:lipid ratio or only submicellar concentrations of detergents throughout purification (30, 56). Uncovering such intrinsic activities presents several problems. First, one must decide for which activities to test. Such experiments may require a considerable effort, because in such analyses *negative results mean nothing*, and because the activity one is searching for may be rather specific. For example, it has been proposed that a serine protease is activated as an early consequence of aggregation (see below). This could be analogous to the mechanism of activation of C1 where C1r becomes a protease. However, C1s is virtually the only known substrate for this enzyme! Therefore, the protease activity of the receptor for IgE, if it exists, could be missed entirely without the appropriate substrate. Furthermore, the intrinsic activity sought for may require the receptor (and its substrate, if there is one) to be incorporated in a bilayer structure—possibly in a bilayer whose total concentration of proteins is similar to the native one. Some general aspects of this latter problem are discussed in Grasberger et al (104). So far no evidence for an intrinsic function of the receptor has been presented.

EXTRINSIC ACTIVITIES The receptor, like IgE, may have no intrinsic function, and a distal component may be the one activated by interaction with the aggregated receptor. We have termed this an *extrinsic function* of the receptor (6). There are a variety of ways to find evidence for such components. Methods such as radiation inactivation and measurement of rotational or translational diffusion have the advantage of being applicable

to intact systems—even living cells—but can give only very indirect information. Direct methods involve trying to isolate a complex composed of the receptor and the relevant molecule, by maintaining the interactions with the use of gentle procedures for purification or covalent cross-linking reagents. Currently two candidates exist for molecules that interact with the receptor (see below), though it is not known whether this interaction is due to some intrinsic action of the receptor upon them or simply to aggregation of the receptor per se.

INITIATION AND PERSISTENCE OF PERTURBATIONS We comment on one additional general aspect of the role of aggregation: that is, whether a particular perturbation, once initiated, can persist in the absence of aggregation of the receptor. Only the very late stages of internalization of the receptor show this property. Thus, whatever the mechanistic role of the aggregation may be, it, rather than some later step, appears to be the time-dependent one for virtually all receptor-mediated events. However, there are the perturbations that, although initiated by and dependent upon aggregation throughout their own lifetime, appear not to persist throughout the lifetime of other aggregation-dependent steps (Table 2). The role of such phenomena is unclear. Whereas results with various inhibitors suggest that at least some of these perturbations play an essential role, they clearly

Table 2 Early perturbations stimulated by aggregating the receptor for IgE

Perturbation	Initiated	Lifetime	Dependent on extracellular Ca^{2+}
Morphological changes in plasma membrane	Very early	Throughout secretion	No
Immobilization of receptors	Very early	Throughout secretion	No
Internalization of receptors	Delayed	Throughout secretion	No
Activation of protease	Very early	Uncertain	No
Activation of methyl transferase	Very early	Brief[a]	Partial[a]
Rise in cAMP	Very early	Brief	No
Hydrolysis of PIs	Early	Throughout secretion	Yes[b]
Opening of channels for Ca^{2+}	Early	Throughout secretion	Yes

[a] Refers to incorporation of counts into phospholipids.
[b] See text. There are some data which suggest otherwise.

cannot simply be the initial step in a single linear sequence of events. They could, however, represent an essential, parallel stimulus.

EARLY EVENTS INITIATED BY THE RECEPTOR

Changes in Plasma Membrane and in Mobility and Distribution of Receptor

CHANGES IN THE PLASMA MEMBRANE Morphological changes in the plasma membrane of cells that were reacted with a variety of ligands have been documented in several systems (105–108). Similar events have recently been documented by Pfeiffer et al on RBL cells, after aggregation of receptor-bound IgE (109). Prior to stimulation the surface of these cells shows numerous microvilli (109, 110), but within 30 sec after aggregating the cell-bound monomeric IgE, the surface becomes highly lamellar. If the aggregation is induced by a multivalent hapten-protein conjugate the original morphology of the membrane is restored within minutes after the addition of hapten. As little as 5 ng of antigen—a dose that produced maximal secretion but only after 30 min—produced maximal morphological changes in seconds (109). Other changes that occur equally rapidly include a dramatic spreading of the cells and increased areas of adhesion to the substrate. A generalized increase in fluid pinocytosis occurs somewhat more slowly. All these events can proceed in the absence of added Ca^{2+} in the medium—a medium in which exocytosis does not occur. Even in the presence of Ca^{2+}, neither the morphological changes nor exocytosis occur if the cells are stimulated while in mitosis. This is true despite the fact that the internalization of the aggregated receptors (111) as well as the receptor mediated influx of Ca^{2+} (112), occurs normally.

CHANGES IN MOBILITY OF RECEPTORS Earlier studies using fluorescent labeling techniques and light microscopy, or ferritin-labeling techniques and electron microscopy (68, 113–119), showed that the diffusely distributed receptors for IgE, like many other membrane proteins, can be clustered with a suitable multivalent reagent. At least in those analyses that involved minimal perturbation of the cells (68), the involved receptors for IgE appeared to be independently mobile. Photobleaching techniques confirmed these observations and showed that the translational diffusion coefficient—2×10^{-10} cm^2 s^{-1} (117)—was similar to that found for other membrane proteins when these were studied with probes that did not aggregate them (120). Examination of the rotational dynamics and electrokinetic mobility of unliganded receptors and of receptors liganded with monomeric IgE has led to similar conclusions (120a, 120b).

Menon et al (98, 121) have extended such photobleaching studies on RBL

cells, using small preformed oligomers of IgE, as well as the "A_2" monoclonal anti-IgE antibody (88) which, as noted above, primarily forms dimers at low concentrations and larger aggregates only at high ratios of antibody to IgE. On a time-scale similar to that observed for the morphological changes in the plasma membrane, substantial decreases occurred in the recovery of fluorescence in the bleached areas—most likely due to immobilization of the receptors. This immobilization occurs in the absence of added Ca^{2+} in the medium and even at sharply reduced levels of cellular ATP. Dimers of IgE, which induce virtually no exocytosis in these cells, also do not induce immobilization. If D_2O is added, the cells do show a modest amount of release and a corresponding immobilization (121). Thus, a close correlation exists between the degree of immobilization of receptors, biochemical perturbations such as the influx of Ca^{2+}, and the breakdown of phosphoinositides (see below), and secretion. Because all of the latter processes require added Ca^{2+} and normal levels of ATP, it is apparent that they cannot account for the immobilization. It is more difficult to prove the opposite, i.e. that the immobilization is a *necessary* antecedent for the other changes.

Suggestive data implicate an interaction of the aggregated receptors with cytoskeletal elements during immobilization. First, immobilization is not seen on the blebs of plasma membranes with which these investigators have recently worked (98). If anything, the mobility of unaggregated receptors on these blebs is increased, so that aggregation of receptors, which should proceed normally on them, appears, in and of itself, insufficient to explain the immobilization. Such blebs are, however, depleted of filimentous F-actin (122). In normal plasma membranes, a reversible attachment of aggregated receptors to actin has been implicated because of partial colocalization of fluorescently labeled clusters of IgE and phallacidin (see below). More direct evidence for such an interaction has also been obtained. If cells are reacted with a covalent cross-linking reagent prior to solubilization with detergent, the aggregated receptors remain in the detergent-insoluble pellet (26, 123)—a pellet which contains cytoskeletal elements.

The process by which aggregated receptors could become attached to cytoskeletal structures is unclear. Indirect evidence for an active process comes from the observation that phorbol esters can induce internalization of membrane proteins (124–28) including the receptor for IgE (K. Furuichi, C. Isersky, personal communication). The phorbol esters probably do aggregate the receptors directly. It is more plausible that they induce covalent changes in one or more cellular components involved in the interaction between the membrane protein and cytoskeletal components, either by activating protein kinase C (128) or by some other mechanism. Nevertheless, because the immobilization of the receptors for IgE (as well as

their coalescence and internalization; see below) is readily reversed by disaggregating them, it is likely that the normal (nondrug-induced) process is passive and based upon multivalent interactions.

Interaction with cytoskeletal components is also implicated in the clustering of aggregated receptors. As noted previously, Holowka & Baird and their colleagues (101) noted that when receptors are aggregated even with relatively small oligomers of IgE, patches containing thousands of receptors gradually form. Such clustering is not observed on the actin-depleted blebs, even when the latter are still attached to cells on which unblistered portions of membrane exhibit large-scale aggregation. These latter clusters appear to be enriched in actin, as assessed by staining with phallacidin (98).

INTERNALIZATION AND REEXPRESSION OF RECEPTORS

Effect of Monomeric IgE When the surface proteins of RBL cells are oxidatively iodinated, e.g. with ^{125}I, and the cells reacted with ^{131}I-labeled IgE at various times thereafter, a fall in the ratio of ^{125}I to ^{131}I is observed in the IgE-receptor complexes subsequently isolated (66, 129). Since any increase in the total number of IgE-binding sites is too small to account for the change in the ratio, the simplest interpretation is that labeled receptors are progressively degraded and replaced by new unlabeled receptors. Assuming such a mechanism, the half-life of the unliganded receptors has been calculated to be 8–12 hr (66, 129). If a similar experiment is conducted, but the cells are exposed to the IgE immediately after labeling and at the start of the incubation, the receptors liganded with IgE and the bound IgE are degraded more slowly. The latter finding agrees with the observation that in cells reacted with radiolabeled IgE and examined by electron-microscopic autoradiography, the retained radioactivity is observed on the surface of the cell only (73). One explanation for all of these results is that whereas unliganded receptors are internalized and degraded, receptors liganded with IgE are not internalized. If so, then the binding of monomeric IgE has some significant effect on the receptor and on its interaction with other cellular constituents. An alternative explanation should be considered. This posits that the binding of IgE produces no significant change in the receptor; the liganded and vacant receptors alike are internalized and exposed to lysosomes. However, just as the receptor can protect bound IgE from being hydrolyzed by exogenous proteases (47), so the bound IgE can protect the receptor from being degraded (28). Such undegraded IgE-receptor complexes would then recycle to the plasma membrane. That receptor-IgE complexes once internalized can be reexpressed (see below) shows that such a pathway exists. The failure to observe internalized monomeric IgE-receptor complexes in the electronmicroscopic studies (73) could be explained quantitatively. At any instant in time only a small

fraction of the total IgE bound to a normally cycling receptor might be in the interior, and thus it could escape detection.

That RBL cells incubated with IgE show an increase in total receptors (66, 129) could be related to the observation that basophils from individuals with high levels of serum IgE have increased numbers of receptors (130). Possibly, the normal mechanism by which the receptors/cell is controlled is simply a balance between degradation and biosynthesis, rather than a more elaborate counting mechanism.

Effect of oligomeric IgE If cells are reacted with preaggregated IgE, or the monomeric IgE on their surface is aggregated with an appropriate multivalent antigen, the receptors, the IgE, and, if present, the antigen are fairly rapidly internalized. This phenomenon has been examined in detail by Isersky and her collaborators (119, 131–135). Their principal findings are: (*a*) Dimers of IgE provide a sufficient stimulus for internalization. Even on RBL cells, where dimers induce little secretion (especially in the absence of D_2O) and exhibit minimal immobilization (see above), the process proceeds expeditiously although not as rapidly as when trimers or higher oligomers are tested (119). (*b*) The internalized covalent oligomers and the receptors to which they are bound are not reexpressed (119). (*c*) The kinetics of internalization are similar on rat peritoneal mast cells and the RBL cells ($t_{1/2}$ 3–5 min), even though the rate of secretion by the mast cells is ~20-fold greater than that by RBL cells (131). (*d*) As is true of the morphological changes in the membrane and the immobilization of receptors described above, internalization proceeds as rapidly in the absence of added Ca^{2+} as in its presence (131). (*e*) The process is incomplete, with 40% or more of the IgE remaining on the surface (119, 131, 132). (*f*) A fraction of vacant receptors, or receptors liganded with monomeric IgE, transiently "coendocytose" when receptors aggregated with IgE are internalized (119). The cointernalization is not totally independent of added Ca^{2+}, and several inhibitors of exocytosis ablate cointernalization of monomeric receptors without affecting internalization of aggregated ones (133). Peculiarly, the fractional coendocytosis is relatively insensitive to the ratio of monomeric to aggregated receptors. The process appears to be specific in that internationalization of monomeric IgE-receptor complexes is not observed even when the receptors for IgG on the same cells are extensively aggregated. These results suggest that at least a proportion of the monomeric receptors may interact with aggregated receptors of the same type (134). A similar conclusion was reached by the Ishizakas on the basis of quite different methods (118). The failure to observe such interactions when copatching or immobilization were examined (68, 98, 117) may have resulted from methods that were less sensitive and that therefore failed to detect

small amounts of such interactions. (*g*) Even at fairly late stages of internalization induced by a multivalent hapten-conjugate, the process can be reversed by addition of hapten (135). Thus, internalization, like so many other phenomena we shall review, is in large part maintained only so long as the receptors remain aggregated.

Relationship to secretion Since the morphological changes in the plasma membrane and the changes in mobility and distribution of the receptors are manifested in the absence of added Ca^{2+}, they must be initiated independently of other early events, such as the influx of Ca^{2+} and the hydrolysis of the phosphatidyl inositides, which require added Ca^{2+} (see below). It is unclear, however, whether the former are parallel phenomena or early steps in the sequence leading to secretion. This question is difficult to explore because most methods provide average data for the entire population of receptors, whereas it is known that aggregation of only a tiny fraction of receptors is sufficient to obtain a near-maximal response of the cells (see above). One experimental finding—but only one—suggests that at least the internalization of the aggregated receptors is not required: When the surface IgE is aggregated by concanavalin A attached to large beads, localized degranulation is observed (102). A test of whether aggregated, internalized receptors are competent to induce secretion could be performed with an impermeant hapten.

Activation of Proteases

Over 20 years ago Austen & Brocklehurst reported that inhibitors and substrates of proteolytic enzymes inhibited secretion by lung mast cells from guinea pigs (136). This basic observation has been reproduced many times with a variety of mast cells, with basophils, and with RBL cells, and with many different inhibitors and substrates that interact with serine proteases (reviewed in 8). Some confusion arose because there appear to be two stages of secretion sensitive to such inhibitors, but it now appears clear that one of these is at an early step. Thus, all of the biochemical changes we describe in subsequent sections appear to be blocked by these inhibitors. Whether the inhibitors also block the morphological changes of the plasma membrane and the changes in mobility and distribution of the receptor that occur upon aggregation of the latter has not been reported. However, preliminary data on RBL cells (K. Furuichi, C. Isersky, unpublished results) suggest that internalization is not inhibited by doses of phenylmethylsulfonyl fluoride that would be expected to block secretion.

The inhibitors of proteases are only effective if they are present during the aggregation of the receptors. If the cells are reacted with the inhibitors and washed prior to stimulation, the receptor-mediated changes occur nor-

mally. This implies that aggregation of the receptors is required to activate a protease which then becomes sensitive to the inhibitor. This protease has been dubbed E^r (137). Evidence exists for an analogous entity (E^d) the receptor-mediated activation of which is required for receptor-induced desensitization (see below) (137, 138). The latter enzyme presumably has a lower K_I for the inhibitors than E^r, since *low* doses of inhibitors (10–40 nM in mouse mast cells) can block desensitization (induced by aggregating receptors in the absence of Ca^{2+}) without blocking the release that occurs when Ca^{2+} is subsequently restored (137, 138). This also indicates that E^d can be activated in the absence of Ca^{2+}. Similarly, activation of E^r appears to be Ca^{2+} independent. Thus, if the receptors are aggregated in the absence of Ca^{2+} and in the presence of *high* doses (10^{-4}–10^{-3} M) of inhibitor and the cells then washed prior to addition of Ca^{2+}, the expected secretion is not observed. Presumably, the receptor-activated E^d and E^r were both inactivated by the inhibitors during the incubation in the Ca^{2+}-free medium. These results are consistent with receptor-mediated activation of two discrete proteases as an early event.

That exogenously added proteases can induce Ca^{2+}-dependent release (139 and references therein) is consistent with such a model, but of course this result can also be explained otherwise. Although it would be interesting to know if exogenous proteases also initiate some of the biochemical changes induced by aggregation of the receptors for IgE, it is difficult to conceive how their use can provide insight into the nature of the putative endogenous enzyme(s). The substantial concentrations of inhibitors that are required to inhibit E^r (see above) make it impractical to use affinity-labeling techniques with radioactive inhibitors to try to identify the enzyme, although this approach might work for E^d. Identifying the molecular basis for the phenomenological observations is a major experimental challenge.

Activation of Methyltransferases

Activation of methyltransferases as an essential step in IgE-mediated secretion by mast cells was first reported by Hirata & Axelrod (140). We discuss these and related studies in some detail, first because it has been proposed that methylation may be one of the earliest steps in secretion, and second because of the controversy that surrounds these findings. To evaluate the experimental data, the metabolic pathways must be understood and some of the methodological problems in exploring them appreciated. We divide our discussion into two sections. We first consider the evidence that methyl transferases are activated by the receptor. We then analyze the evidence that the relevant substrates are phospholipids.

GENERAL ASPECTS Methylation reactions control many biochemical pathways. Discrete methyltransferases catalyze the transfer of a methyl group to

either phospho- or neutral lipids, to proteins, or to DNA, or RNA (t, r, and m) (141, 142). These modifications are thought to be important in cellular functions such as chemotaxis, transport, and gene expression. Despite the wide range of substrates, all methyl transfer enzymes use s-adenosylmethionine (AdoMet) as the methyl donor and all are competitively inhibited by s-adenosylhomocysteine (AdoHcy), the demethylated product of AdoMet (Figure 2). It should be noted, however, that AdoMet is also a substrate for AdoMet decarboxylase (143) and interacts with a variety of other proteins (reviewed in 141).

Since the K_I for AdoHcy of various transmethylases vary over a 1000-fold range (144), the activity of a particular enzyme will depend on the relative concentrations of AdoHcy and AdoMet, and on the ratio of its K_m for AdoMet to its K_I for AdoHcy. Certain of these enzymes may thus be regulated by the intracellular concentration of AdoHcy via AdoHcy hydrolase.

The latter enzyme is the only one that metabolizes AdoHcy in eukaryotic cells. It cleaves AdoHcy into adenosine and homocysteine (Figure 2)—a hydrolysis that is readily reversible. In fact, the equilibrium constant probably favors synthesis, but under normal conditions adenosine is rapidly metabolized by adenosine deaminase and the AdoHcy hydrolase reaction is pulled towards hydrolysis.

Many investigators use pharmacological agents to raise the intracellular levels of AdoHcy, in order to establish that a methylation reaction is involved in a particular cellular function. Addition of adenosine plus homocysteine increases AdoHcy by driving AdoHcy hydrolase in the synthetic direction. This effect can be potentiated and prolonged by

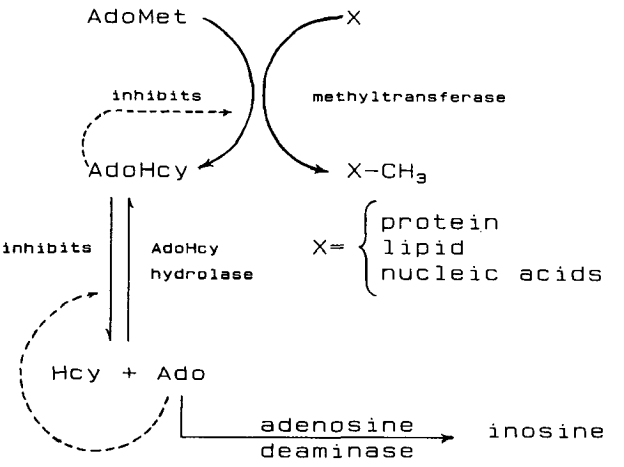

Figure 2 Enzymes that control methyl transferase reactions.

inhibitors of adenosine deaminase. In addition, there are analogues of adenosine and AdoHcy that inhibit AdoHcy hydrolase and cause an elevation of AdoHcy. Several adenosine analogues are substrates for AdoHcy hydrolase. Administering these compounds, along with homocysteine, or an analogue thereof, results in the buildup of analogues of AdoHcy that also inhibit transmethylases (141, 142).

EFFECTS OF INHIBITORS Inhibitors of methyltransferases block IgE-mediated secretion by a variety of mast cells as well as by RBL cells (8, 145). Evidence that early receptor-mediated events are affected is based on the concomitant inhibition of (*a*) stimulated methylation of phospholipids, (*b*) the early rise in cAMP, (*c*) the influx of ^{45}Ca, and (*d*) an absence of an effect on histamine release stimulated by Ca^{2+} ionophores (146). Numerous studies of the effects of inhibitors of methylation have been reported. For example, human lung mast cells were incubated with 3-deaza-adenosine and homocysteine (thiolactone (147). These should induce AdoHcy hydrolase to synthesize 3-deaza-adenosine-homocysteine, an inhibitor of methyltransferases. That action on the latter was responsible for the observed inhibition of the cellular activities was strongly supported by the counter-inhibitory action of AdoMet (148, 149). Although higher doses of the inhibitors were required to diminish histamine release than to diminish the rise in cAMP and the influx of ^{45}Ca (Figure 6 in 147), this can be easily rationalized. Only a fraction of the receptors must be aggregated to produce maximal secretion (see above), whereas at least some of the biochemical perturbations that are stimulated by the receptors are directly proportional to the number of aggregates formed (see below). Thus, under conditions where most of the receptors are aggregated, it is likely that a receptor-mediated early event would have to be substantially inhibited before a decrease in secretion was evident. Studies on RBL cells have similarly shown that inhibitors of methyltransferases cause concomitant inhibition of influx of ^{45}Ca and of secretion (150).

Further evidence that the action of the inhibitors of methyltransferase is specific comes from studies of their effect on desensitization, which we discuss more fully below. That the inhibitors can actually prevent inactivation of the cells under conditions where secretion by untreated cells is inhibited (137) is reassuring.

If the aggregation of receptors leads to activation of methyltransferases, one might be able to observe this indirectly by finding some decrease in their substrate, AdoMet. The published data on such experiments are contradictory. Moore et al failed to observe any decrease in RBL cells (151), whereas Ishizaka et al (152) saw a 12-fold drop in incorporated radioactive AdoMet with mast cells. The latter is rather surprising since it implies that

upon triggering of the receptors for IgE, the cells metabolized over 90% of their pool of AdoMet in ~15 sec!

METHYLATION OF PHOSPHOLIPIDS Hirata et al first reported that bridging the cell-bound IgE on mast cells with concanavalin A promoted methylation of phospholipids (153). This group investigated the phospholipid methyltransferases in several systems. From studies on red blood cells, they proposed that methyltransferase I, in the inner leaflet of the membrane, monomethylated phosphatidylethanolamine which, upon flipping to the outer leaflet, was further methylated by a methyltransferase II located there (154). Although this pathway is a physiologically minor one for generating phosphatidylcholine, they proposed that activation of these enzymes is a critical event in the stimulation of cells by several different receptors (140).

Before reviewing the findings on mast cells and RBL cells, two methodological points should be stressed. Virtually all the studies where changes in methylation of phospholipids have been claimed involve simply the measurement of chloroform extractable counts from cells whose AdoMet pool has been labeled by incubation in [methyl^{3}H] methionine. Although it has been stated that the phospholipids in the chloroform phase showed "a marked" incorporation of radioactive methyl groups (152), the fraction of the radioactivity in such extracts is not regularly assessed. Moore et al recently showed that as little as 30% of such counts may be associated with phospholipid, the remainder being in undefined moieties (151). It should also be noted that in experiments with inhibitors, only a single time point is routinely reported so that one cannot determine whether incorporation of methyl groups into chloroform-soluble moieties was reduced or simply slowed.

Ishizaka and her colleagues regularly observe incorporation in mast cells whose receptors for IgE have been aggregated in a variety of ways (8). Recently they showed that interrupting the aggregation diminished the incorporation (149), but a dose response curve relating the number of receptors aggregated to the extent of incorporation has never been reported.

The time course of incorporation is curious. It begins promptly, peaks at 15 sec after addition of stimulant and returns virtually to baseline in 30 sec to 2 min. Since their earlier data showed rapid marked depletion of radioactive AdoMet (above), one can rationalize a failure to see a further rise in radioactivity, but the decline suggests virtually immediate breakdown of the chloroform-soluble moieties. There is nothing in the current models that suggests a mechanism or role for this rapid change. If the radioactively labeled AdoMet is rapidly metabolized, this does not of course mean that there is a net change in total cellular AdoMet since this

might be continuously replenished. If so, then similarly any methylated products derived from the newly synthesized AdoMet would be unlabeled. Therefore, these experiments are not informative about any net changes in phospholipids as a consequence of aggregation of the receptors. In analogous experiments on RBL cells, Crews et al presented similar results, although the kinetics of incorporation, of ^{45}Ca uptake and of secretion were all substantially slower (150). Using nominally the same line of cells, Moore et al (151) failed to observe stimulated incorporation of methyl groups, although IgE-mediated influx of $^{45}Ca^{2+}$, release of arachidonic acid, breakdown of PIs (see below), and secretion were substantial. They used conditions that gave optimal incorporation of [methyl^{3}H] methionine into AdoMet but observed no enhanced incorporation of counts into individual phospholipids as assessed by high pressure liquid chromatography. As already noted, they also failed to see any enhanced metabolism of AdoMet.

The basis for this discrepancy is unclear. Clearly, it is more reliable to quantitate changes in the specific phospholipids directly than to rely simply on counts extracted in the chloroform phase. It would also be useful to see if the differences in the specific methods used were important (cf 150, 151). Finally, it is regrettable that in the study by Moore et al, the effects of inhibitors were not tested since, as we emphasize here, the question of whether methyltransferases are involved and if so, which substrates they modify, are separate issues.

Ishizaka tried to define more precisely which of the phospholipid methyltransferases were involved (148) by exploiting the finding of Hirata & Axelrod that transferase I requires Mg^{2+} but not Ca^{2+} (155). Ishizaka reported that when mast cells were incubated in the presence of Mg^{2+} and EGTA, methyl groups were incorporated only into phosphatidylethanolamine as expected. She also observed that receptor-mediated incorporation of ^{32}P into phosphatidic acid, phosphatidylcholine, and phosphatidylinositol was unabated. It should be noted that this is the first report to claim that inhibitors of methyltransferases such as 3-deaza-adenosine inhibit receptor-mediated breakdown of PIs.

If an early consequence of aggregating the receptor for IgE is activation of phospholipid methyltransferases, it might be expected that this could be observed with preparations of plasma membranes. Positive results have been reported for membrane preparations from rat mast cells (156) but are difficult to evaluate. First, the results are reported in dpm, whereas comparable experiments on mast cells are reported in pmol, and there is insufficient information to convert one unit into the other. Second, the incorporation by the intact cells was measured at 15 sec as usual, whereas that by the membranes was determined at 60 min. Thus, it remains to be

proven that such preparations show a significant amount of activity relative to intact cells.

McGivney et al reported an innovative approach to this subject (157). They treated RBL cells with a powerful mutagen and selected clones grown from the small number of surviving cells which (*a*) bound IgE, (*b*) failed to secrete in response to aggregation of cell-bound IgE, (*c*) did secrete in response to ionophore, and (*d*) were deficient in either phospholipid methyltransferase I or II. When a clone missing enzyme I was fused with one missing enzyme II, IgE-mediated secretion was partially restored. Their strategy is modeled on one used to select specific bacterial mutants. However, with bacteria, specific metabolic intermediates can be used to growth-select appropriate auxotrophs. This could not be done with the mutagenized RBL cells, so the number of genes mutated in the chosen variants is indeterminate. Many complementation studies would have to be performed to demonstrate that it was loss of the phospholipid methyl transferases that was the only relevant variable.

APPRAISAL After reviewing the published data we conclude that the evidence for involvement of methyltransferases—evidence derived principally from studies with inhibitors—is more solid than the evidence that phospholipids are the relevant substrates. That no increase in methylation of bulk cellular proteins (and polynucleotides) has been observed (150, 152) could simply reflect inadequate sensitivity or unrecognized degradation. As noted above, even the incorporation into chloroform-soluble moieties is relatively transient. It is also worth noting that whereas the results with inhibitors suggest that methyltransferases are involved in early steps that precede the rise in intracellular Ca^{2+}, the methylation of phospholipids could involve much later steps, unless it can be proven that lipids are the only substrates on which the stimulated methyltransferases are acting. In the end, proof for the role of methyltransferases will have to await information about what their activation accomplishes in molecular terms. There are no data whatsoever on this matter.

Activation of Adenylate Cyclase

PRINCIPAL FINDINGS Aggregation of the receptor on mast cells initiates a rapid rise in cAMP (8). The nucleotide increases to a maximum in less than 30 sec and returns to basal levels in ~2 min. The rise in cAMP begins a little later than the stimulated incorporation of methyl groups (see above), and despite earlier reports to the contrary (158), recent studies show that it can be blocked by inhibitors of methyl transferases (8). Since the rise in cAMP occurs in the absence of extracellular Ca^{2+} (137) and prior to the stimulated uptake of $^{45}Ca^{2+}$ (seen in the presence of Ca^{2+}) (148), it appears to be an

early event initiated by aggregation of the receptors. As with other receptor-mediated events, the apparent stimulation of adenylate cyclase is blocked by protease inhibitors (8). Indirect evidence for a receptor-mediated increase in cellular cAMP has also been obtained by demonstrating increased levels of cAMP-activated protein kinases (159, 160).

Since adenylate cyclase is a plasma membrane protein, one anticipates that the receptor-mediated stimulation of this enzyme should also be observable on isolated membranes. Although positive results have been claimed (156), the stimulation appeared to be substantially less than that observed on intact cells. For the membranes, the rise in cAMP took $\sim$60 min to reach a maximum (cf above), and we calculate that, per cell equivalent, the stimulation of the cyclase in the membranes was only $\sim 2 \times 10^{-5}$ of that observed in intact cells! Even if we correct for the fact that binding of IgE to the membranes was only 0.3% of that expected for intact mast cells, the recovery of activity was only $\sim$0.6%.

SIGNIFICANCE The significance of the apparent stimulation of adenylate cyclase is unknown. That no rise in cAMP is seen in receptor-mediated secretion from RBL cells (145) suggests that the rise observed in mast cells may be incidental rather than essential. Possibly, it is related to receptor-mediated inactivation (desensitization) (below), since mast cells are much more readily desensitized than RBL cells. Furthermore, when intracellular cAMP is increased artificially, secretion of mediators is frequently inhibited rather than enhanced, although discrepant results have been reported (161). Perhaps the rise in cAMP represents simply a side effect of the interaction between the receptor and GTP-binding regulatory proteins—an interaction that has been proposed to mediate receptor-initiated activation of phospholipase C (see below). Studies analogous to those with inhibitors of methyl transferases that have strengthened the case for the importance of those enzymes (see above) would be useful for evaluating the significance of the stimulation of adenylate cyclase.

Breakdown of Phosphatidyl Inositides (PIs)

The breakdown of PIs in calcium-dependent activation of cellular events by membrane receptors is receiving increasing attention (162–164). After outlining the elements of this system, we will briefly describe older data on mast cells and then focus on newer studies on RBL cells.

METABOLISM OF PIs Phosphatidyl inositol (PI) and its phosporylated derivatives phosphatidyl inositol-4-phosphate (PIP) and phosphatidyl inositol-4,5-bisphosphate (PIP_2) are ubiquitous components of eukaryotic membranes; in mammalian cells they constitute 5–7% (PI), 1% (PIP), and 0.4% (PIP_2) of the total phospholipids (164). PI is synthesized from the

glycerol backbone in the endoplasmic reticulum, from which it is transferred to the plasma membrane. Because the PI kinases as well as other relevant enzymes appear in the cytoplasm (as well as on membranes), it is assumed that the PIs are chiefly localized in the inner leaflet of the plasma membrane (165, 166).

In some instances (167) the receptor-mediated release of the inositide moiety can be catalyzed by a phospholipase D (which, therefore, also generates phosphatidic acid), but in most systems it is phospholipase C (which generates diacyl glycerol and inositol phosphates) whose action is enhanced. Soluble cytoplasmic forms of the latter enzyme have been isolated from liver and from sheep seminal vesicles (168, 169). The enzymes from the latter show greater activity towards PIP and PIP_2, when these lipids are presented in vesicles; and the vesicles hydrolyze them at submicromolar concentrations of Ca^{2+} (170). Whether membrane-bound phospholipase C activity (171–173) represents a distinct entity is uncertain. It is also unclear whether the role of receptors in initiating breakdown of the PIs is to activate a membrane-bound enzyme, to recruit cytoplasmic enzyme to the membrane, or simply to mobilize membrane PIs making them more susceptible to hydrolysis.

Despite the ability of variations in Ca^{2+} concentrations to modulate the activity of phospholipase C (170, 174, 175), current models of receptor action postulate that this pathway is activated directly. Receptors are thought to stimulate the enzyme—possibly through an intermediary GTP-binding regulatory protein (below)—releasing inositol phosphates that in turn release Ca^{2+} from intracellular stores (163).

OLDER REPORTS ON MAST CELLS Early studies reported turnover of PI as a consequence of aggregating receptors for IgE on mast cells on the basis of an enhanced incorporation of ^{32}P and/or ^{3}H-inositol into PI, phosphatidyl choline, and phosphatidic acid (176–178). The dose response curve of release of histamine and incorporation of ^{32}P into these lipids correlated well. However, when stimulation was performed in the absence of Ca^{2+}, incorporation of ^{32}P and ^{3}H-inositol into PI persisted whereas secretion did not occur. These studies have methodological drawbacks that were common to a number of early reports in this field: (*a*) The techniques for either extracting or separating the lipids did not permit one to visualize the inositol polyphosphates (179) and (*b*) brief preincubation with the radioactive label (^{32}P or ^{3}H-inositol) and measurements of the lipids after rather lengthy stimulation with the agonist prevented direct observation of the initial stages in the enhanced breakdown. In mast cells the breakdown of the PIs is initiated 5 to 30 sec after activation, and 1 to 2 min later the resynthesized lipids return to their baseline values, increasing further after

longer incubations (180, 181). Short incubation times are particularly important for observing the rapid hydrolysis of the inositol polyphosphates, especially when this is assessed by quantitating the lipids rather than the water-soluble breakdown products. Thus, an enhanced phosphorylation of diacyl glycerol (to phosphatidic acid) is observed in mast cells within 8 sec after activation, whereas labeling of PI and phosphatidyl choline starts only after two min (177).

STUDIES ON RBL CELLS

Correlation with aggregation of the receptors Activation of RBL cells by aggregating the bound IgE with an appropriate antigen increases the level of the inositol phosphates within 10 to 40 sec (182). Experiments with preformed oligomers of IgE showed an excellent correlation between the amount of oligomer bound (and, therefore, of receptors aggregated) and the rise in the inositol phosphates (103). Larger oligomers were more effective than trimers, and trimers much more than dimers—exactly what was observed when secretion and rises in $[Ca^{2+}]_i$ were measured (see below). Similarly, the effectiveness of all of the oligomers was enhanced by D_2O. As was true for the rise in intracellular Ca^{2+}, doses of oligomers above those necessary to achieve maximal secretion further enhanced the breakdown of PIs. Thus, the failure to obtain 100% release of mediators cannot be ascribed to limitations of the metabolism of PIs. Finally, it is noteworthy that—again as was true for the rise in $[Ca^{2+}]_i$—doses of trimer and larger oligomers, necessary to achieve maximal secretion, led to equivalent breakdown of the PIs. Interrupting the aggregation of the receptors promptly halts several receptor-initiated phenomena including secretion, but such protocols have not yet been applied with respect to turnover of PIs.

Characteristics of breakdown products After stimulation, RBL cells exhibit an initial rapid rise in IP_2, followed by a smaller increase in IP_3. Both of the latter reach a plateau value at about 10 to 15 min, whereas the rise in IP, which begins at about the same time as the rise in IP_3, continues virtually linearly for over 20 min (Figure 1 in 182). Since the polyphosphoinositides are present in much smaller amounts than PI (the ratio is about 1:1:20 in RBL cells for PIP_2, PIP, and PI, respectively), it is apparent that there is a substantial preferential breakdown of PIP_2 and PIP. However, it is not clear whether the IP is generated exclusively from the action of phosphatases on IP_3 and IP_2 (as is the case in some systems (183)), or whether it also results from direct breakdown of PI. If the latter is not the case, then only continuous synthesis of PIP and PIP_2 can account for the amount of IP that is generated. Possibly the inhibition observed when the cells are depleted of ATP (184) is due to reduced synthesis of PIP and PIP_2.

In mast cells PIP also decreases, reaching a minimum 5 sec after stimulation and thereafter increasing (apparently at the expense of PI) back to baseline values at about 30 sec (181). In those studies both PIP and PI reached levels higher than those prior to stimulation at 1 and 2 min respectively. No substantial changes in PIP_2 were noted.

Dependence on Extracellular Ca^{2+} The breakdown of PIs initiated by aggregating the receptors for IgE on RBL cells is dependent upon adequate extracellular Ca^{2+}, in apparent contradiction to the situation observed in mast cells (see above). It is possible that the results on mast cells were amplified by a failure to reach isotopic equilibrium during the short incubations employed. The apparent synthesis of PIs may have been relatively minor and cannot be assumed to reflect accurately the extent of breakdown of the PIs. In RBL cells the variation in breakdown as a function of external Ca^{2+} parallels exactly the variation in the rise of intracellular Ca^{2+} and secretion (182). However, ions such as La^{3+} and Zn^{2+} that inhibit the rise in $[Ca^{2+}]_i$ and secretion [presumably by blocking the passage of Ca^{2+} through receptor-induced channels (see below)] do *not* block receptor-mediated stimulation of breakdown of PI. Since under these circumstances the IP_3 generated should release Ca^{2+} from internal stores, it is curious that no change in quin-2 fluorescence was detected (182). Raising the intracellular Ca^{2+} directly with ionophores does not in itself substantially stimulate hydrolysis of PIs.

ROLE OF GTP-BINDING PROTEINS We have already noted that in some systems a GTP-binding regulatory protein has been postulated to serve as a go-between from the activated receptor to the phospholipase C, in analogy to the role such proteins play in receptor-mediated activation of adenylate cyclase (185–188). The evidence is based partly on the effect of GTP or its nonhydrolyzable analogues and partly on the use of toxins such as pertusis, at least one of those actions is on the inhibitory GTP-binding protein N_i. Although effects of both types of reagents have been noted on mast cells, their site of action is by no means certain (189–191).

Pertussis toxin has been reported to inhibit passive cutaneous anaphylaxis in the rat and to inhibit partially IgE-mediated release of histamine from peritoneal rat mast cells in vitro (192). We have seen no effect of the toxin on IgE-mediated turnover of PIs or on secretion from RBL cells, even though ADP-ribosylation of a 41 kd protein was documented (unpublished observations in collaboration with J. Moss). The effect of the toxin on breakdown of PIs *mediated by the receptor for IgE* has not been reported for mast cells. The GTP analogue, Gpp(NH)p, introduced into transiently permeabilized mast cells, causes secretion in the presence of Ca^{2+}, and this reaction is inhibited by neomycin (thought to act on PIs) (179). However, these findings are insufficient to prove the involvement of a GTP-binding

regulatory protein in the breakdown of PIs induced by the receptor for IgE.

We have explored the possibility that the receptor itself might be a member of the family of the GTP-binding regulatory proteins, since there are some obvious similarities between the subunit structure of the receptor and these proteins (185). Comparative peptide-mapping data and our initial analyses of the amino acid composition of the β subunit suggest no such relationship. Nor have we seen consistently positive results on Western blots using antibodies (193) that react with the β chain common to all GTP-binding proteins (J.-P. Kinet, H. Metzger, unpublished observations).

SUMMARY AND FUTURE DIRECTIONS The special feature of the receptor-induced breakdown of PI in RBL cells (but less clearly on mast cells) is its dependence on extracellular Ca^{2+}—a dependence not observed in several other systems. Since the evidence is against the stimulation of breakdown being initiated (or even requiring) a rise in *intra*cellular Ca^{2+} (see below), the high concentration of extracellular Ca^{2+} that is required [$K_m \sim 0.4$ mM (192)] must be for an earlier step. The receptor-induced *formation* of ion channels occurs even in the absence of Ca^{2+}, but the *specificity* of such channels for Ca^{2+} requires concentrations of extracellular Ca^{2+} similar to those required to initiate breakdown of PIs (see below). If these observations are related, then either these latter phenomena share a common Ca^{2+}-dependent antecedent step or one of them precedes the other. If the breakdown of PIs is a necessary antecedent for the initiation of channels specific for Ca^{2+}, this would provide a ready explanation for the existence of the PI pathway in this system.

It should be emphasized that there is still no evidence that hydrolysis of PIs plays a significant role in the cellular functions stimulated by the receptor for IgE. The lack of specific inhibitors of this system forecloses one obvious approach to this question. One substance that does inhibit receptor-induced breakdown of PIs and the rise in cytosolic Ca^{2+} completely (but inhibits secretion only partially) is phorbol myristic acetate (194; M. Beaven, personal communication). This is interesting because this substance stimulates protein kinase C, an enzyme that is also activated by a metabolite of PI (and other phospholipids): diacylglyceride. Whether the receptor-mediated breakdown of PI secondarily stimulates protein kinase C, and how this may relate to other receptor-mediated secretory events, are under investigation.

It will ultimately be important to reconstitute the receptor-mediated hydrolysis of PIs using purified components. As a step in that direction, the demonstration of receptor-mediated breakdown on isolated plasma membranes is important. Although a variety of preparations of such membranes from RBL cells contain phospholipase C activity, we have not observed

stimulation of the enzyme when the receptors are aggregated, even after readdition of cytoplasmic components. Indeed, cells simply permeabilized (with saponin), as gently as possible, lose their responsiveness even though the cytoplasmic contents have not been aspirated from the sample. In experiments on cytoplasmic phospholipase C, the hydrolysis of the PIs is markedly affected by the other phospholipids present in the mixture and by deoxycholate (174, 175). Similarly we and others (195) have found that hydrolysis of PIs in intact cells is exquisitely sensitive to low concentrations of organic solvents. It appears that the topological relationships between the proteins and lipids are critical in these reactions.

Increase in Intracellular Ca^{2+}

OLDER STUDIES (196) Since the observations of Mongar & Schild (197), it has been repeatedly shown that mM concentrations of extracellular calcium are required for optimal receptor-mediated secretion. Because maneuvers that directly raise the intracellular Ca^{2+} induce secretion (198–202), it is reasonable to postulate that the receptors for IgE initiate a transfer of calcium ions from outside the cell to the inside. Aggregation of receptors induces an enhanced uptake of $^{45}C^{2+}$ (150, 203), which supports this proposal. Although this apparently enhanced uptake could be due to a stimulated decrease in efflux, direct assessment of this with RBL cells showed that efflux actually was stimulated, not diminished (204). The enhanced uptake of $^{45}Ca^{2+}$ could also result simply from an enhanced exchange across the membrane without a net increase in cytoplasmic Ca^{2+}, and this possibility cannot be excluded by such studies. Recent investigations have more directly demonstrated a net rise in intracellular Ca^{2+} and provided evidence that this occurs owing to the receptor-induced opening of ion channels in the plasma membrane.

MEASUREMENTS OF $[Ca^{2+}]_i$ The development of the fluorescent tetracarboxylate probe quin-2 by Tsien (205) has permitted rather simple quantitation of intracellular Ca^{2+}, and a detailed examination using this approach with RBL cells has been performed by a group in Cambridge, United Kingdom (151, 182, 206). Measurements on unstimulated cells showed the intracellular concentration of Ca^{2+} to be 104 ± 5 nM, and over brief periods this was essentially independent of extracellular Ca^{2+} in the range 0.05 to 1.0 mM. At very low concentrations of extracellular Ca^{2+} ($< 5\ \mu$M) obtained by EDTA, the $[Ca^{2+}]_i$ progressively decreased, the rate of efflux being ~ 60 amol ions/cell/min; adding back Ca^{2+} to the medium caused a corresponding rate of uptake. This dynamic balance of $> 3.6 \times 10^7$ ions/cell/min, which maintains an intracellular concentration of ~ 100 nM, was resistant to marked decreases in intracellular ATP.

Upon aggregation of the cell-bound IgE with antigen (ovalbumin), or with concanavalin A, a prompt increase in $[Ca^{2+}]_i$ was observed at rates greater than 1 fmol/cell/min under maximal stimulation. This is greater by a factor of two than the rate that can be calculated from the ^{45}Ca measurements of Crews et al on RBL cells (150). Intracellular concentrations up to 12-fold higher than the resting concentrations were observed by Beaven et al (206). The essential features of receptor-mediated increases in $[Ca^{2+}]_i$ were these: (*a*) The increase was only observed at $[Ca^{2+}]_0 > 50\ \mu M$. Whereas the normal influx pathway appeared to saturate at $[Ca^{2+}]_0 > 0.1$ mM, the pathway stimulated by the receptors was saturated only at 10-fold higher concentrations, with an estimated $K_m \sim 0.4$ mM. In studies on rat mast cells, White et al (207) observed an increase in $[Ca^{2+}]_i$ upon aggregation of receptors even in the presence of EGTA. However, in those studies the cells were exposed to 100 μM quin-2 (cf 206), a concentration at which there is evidence of cell cytotoxicity (151). This seriously complicates the interpretability of that study. (*b*) The rise in $[Ca^{2+}]_i$ was directly related to $[Ca^{2+}]_0$ (206). (*c*) After an initial rapid rise, the $[Ca^{2+}]_i$ showed only a slow decrease as long as the cross-linking of the receptors was maintained. When aggregation of the receptors was disrupted, there was a rapid decrease similar to the decrease observed by addition of lanthanide ions or EDTA. (*d*) The enhanced transport of Ca^{2+} into the cell required cellular ATP levels well above those required for the transport of Ca^{2+} into unstimulated cells (see above).

These studies were recently extended in experiments employing covalent oligomers of IgE (103). Such oligomers allow one to vary both the total number of receptors aggregated and the multiplicity of the individual aggregates that form more rigorously than can be accomplished either with antigen or with anti-IgE antibodies. The new studies by Maeyama et al (103) document the relationship between the aggregation of receptors and rises in $[Ca^{2+}]_i$. They also show that the enhanced effectiveness of larger oligomers as inducers of secretion is mirrored by the enhanced increases in the intracellular Ca that they induce. At the higher doses of the larger oligomers, more receptors become aggregated than appear to be necessary to generate the maximal amount of secretion. It is noteworthy that in the studies of Maeyama et al the maximal $[Ca^{2+}]_i$ continues to increase until doses of IgE are reached that saturate the receptors. This differs from the release of histamine but is similar to the hydrolysis of PIs (see above). These findings suggest that the maximal amount of secretion is not limited as a consequence of the mechanism that generates the rise in cellular Ca^{2+}. Similar to all other receptor-mediated events that have been examined so far, D_2O substantially enhanced the uptake of Ca^{2+} (103).

The relatively low extinction coefficient (< 5000) and quantum yield (0.03

to 0.14) of quin2 require that loadings of several tenths of millimolar or more (internal concentration) are necessary to overcome cellular autofluorescence. Such concentrations may perturb cells [e.g. mast cells (207)], buffer transient changes in $[Ca^{2+}]_i$, and may have other undesirable side effects (208). A new generation of indicators (208) have 30-fold brighter fluorescence, exhibit a shift in wavelength (not just an increase in intensity) when they bind Ca^{2+}, and have an increased selectivity for Ca^{2+}. Their development should substantially improve the usefulness of this approach.

DEPOLARIZATION OF PLASMA MEMBRANE POTENTIAL Evidence for a receptor-mediated opening of a channel for Ca^{2+} through the plasma membrane has been sought, using the tetraphenylphosphonium cation as a probe. Equilibration of this ion between the medium and the interior of cells is thought to reflect a combination of the potential difference ($\Delta\psi$) across the plasma and mitochondrial membranes (209, 210). The formation of channels through the plasma membrane that allows the entrance of positive ions diminishes $\Delta\psi$, and phosphonium ions are released into the medium to an extent governed by the Nernst equation: $\Delta\psi = -(RT/F)\ln[(probe_{in})/(probe_{out})]$. Here R is the gas constant, T, the absolute temperature, and F is Faraday's constant. Experimentally, the change in potential can be indirectly assessed by measuring the decrease in cell-associated tritiated phosphonium ions.

Two groups used this approach on RBL cells stimulated with either anti-IgE (211–213) or an appropriate multivalent hapten-conjugate (211). Both groups observed slow ($\sim$30 min) equilibration of the probe, which makes kinetic experiments (212) difficult to interpret. Upon aggregation of the receptor-bound IgE, partial release of the phosphonium ions was observed. Addition of hapten, which did not affect release induced by anti-IgE, led to prompt reuptake of the ions (211).

The results of these studies differed in two important respects. In order to assess the role of the plasma membrane in the receptor-induced effects, both groups sought to eliminate the influence of the mitochondria. Sagi-Eisenberg & Pecht (213) used the protonophore carbonyl-cyanide-p-trifluomethoxy phenyl hydrazone; they observed a 50% drop in the potential of unstimulated cells and little change after aggregation of the receptors. They, therefore, concluded that the changes observed on unpoisoned cells did *not* reflect changes in the plasma membrane, but rather reflected uptake of Ca^{2+} by the mitochondria, secondary to an increase of that ion in the cytoplasm. Kanner & Metzger (211) blocked the mitochondrial respiratory chain with rotenone and the proton-dislocating adenosine triphosphatase with oligomycin. They observed an $\sim$20% drop in the baseline potential. Notably, the *relative* decrease initiated by aggregation

of the receptors was still observed. High concentrations (100 mM) of the probe—which preferentially collapse the mitochondrial potential difference—gave similar results. They, therefore, concluded that the receptor-induced changes were *principally* the result of a change in the plasma membrane potential.

A further difference was in the influence of external Ca^{2+}. Eisenberg & Pecht observed a parallel decrease in receptor-mediated depolarization and secretion (212), whereas Kanner & Metzger observed comparable receptor-induced depolarization in the presence or absence of mM Ca^{2+}, even though in the latter medium, secretion was totally blocked (211).

The independence of depolarization from the concentration of external Ca^{2+} led the latter workers to suggest that the putative plasma membrane channel could pass Na^+ at low concentrations of Ca^{2+}. This was experimentally verified by studying the uptake of $^{22}Na^+$ (214). The authors concluded that the plasma membrane channels are induced by aggregation of the receptors, regardless of the concentration of external Ca^{2+}, but become specific for Ca^{2+} only at mM concentrations of the latter (214). Their conclusions are consistent with the "direct" measurements of cytoplasmic Ca^{2+} using a fluorescent probe (206), as well as with the characteristics of the putative channel protein (see below). The latter approaches can yield more easily interpretable data. It seems, therefore, more productive to focus future efforts on these more direct measurements, rather than to try to unravel the cause of the contradictory findings from studies on the membrane potential.

MOLECULAR BASIS OF CHANNEL ACTIVITY In a series of provocative papers (215–220) Mazurek, Pecht, and their collaborators characterize a plasma membrane protein from RBL cells that they propose is the Ca^{2+}-channel-forming protein whose activity is initiated by aggregation of the receptors for IgE. The basis of their work is the drug cromolyn—a drug known to inhibit IgE-mediated secretion from mast cells. One of the mechanisms that has been proposed for the action of this drug is that it interferes with the receptor-mediated gating of Ca^{2+} through the plasma membrane. The correctness of this proposal forms the conceptual basis for interpreting the results of Mazurek et al. It should be noted, however, that the mechanism(s) of action of cromolyn is by no means certain. Cromolyn can have a variety of actions and, indeed, after a thorough review of the subject (196), Pearce concluded that "the calcium gating mechanism must now be rejected and alternative explanations for . . . [its] . . . action should be sought."

The principal findings of Mazurek et al are as follows: (*a*) Rat peritoneal mast cells and RBL cells bind beads conjugated with cromolyn, in a Ca^{2+}-dependent manner (215). (*b*) A surface membrane protein (M_r 60,000 in

unreduced gels) can be isolated from detergent lysates of RBL cells using the ligand-binding properties of the protein and either immunoprecipitation or affinity chromatography (216). Substantial amounts of the protein can be isolated. If we assume an overall yield of ~30%, their data (219) suggest ~6 × 10^5 copies/cell, i.e. an amount at least equal to the number of receptors on these cells (*c*) Using a cell sorter, variants of RBL cells can be isolated that are deficient in binding cromolyn and in IgE-mediated influx of Ca^{2+} and secretion (217). These variants appear to have a normal capacity to bind IgE and to respond normally to ionophore. (*d*) If such variants are fused with envelopes of Sendai virus implanted with isolated cromolyn-binding protein, virtually full IgE-mediated influx of Ca^{2+} and secretion are restored. These activities can be blocked by ascitic fluid said to contain monoclonal antibodies to the cromolyn-binding protein (218). From the sigmoidal-dose-response data the authors calculate that two molecules of the protein are required to generate a unit response (218). (*e*) If isolated cromolyn-binding protein is reconstituted into planar bilayers and reacted with a monoclonal antibody said to initiate degranulation of RBL cells, conductances can be measured in the presence of Ca^{2+} and Na^+ (219). The conductances are similar to those observed when receptor-bound IgE in planar bilayers prepared from the plasma membranes of RBL cells is aggregated. In either case, cromolyn blocks the conductances. The flux of either Na^+ (at low concentrations of Ca^{2+}) or Ca^{2+} (at mM concentrations of Ca^{2+}) is consistent with the observations of Kanner & Metzger referred to above (214). The findings of Mazurek et al are obviously of considerable interest; they report the first direct evidence for a cellular component that may interact with the receptor and that performs one of the critical receptor-mediated events.

There are, however, several aspects of the published work that require clarification: (*a*) Most, though apparently not all, workers have failed to observe any reproducible inhibition of degranulation by RBL cells with cromolyn, and indeed, Mazurek et al have published only minimal data in this regard. (*b*) The published method for preparing the conjugates of cromolyn—an essential procedure without which none of their studies can be reproduced—does not work in our laboratory or apparently in the hands of others. An analysis of the derivative of cromolyn from which the conjugates were made has not been given. (*c*) The evidence that the cromolyn-binding protein used in the various experiments is pure is, as yet, inadequate. Indeed, close inspection of their gels shows a disturbing similarity between their principal component and serum albumin, and they themselves appear to be uncertain about the significance of a variably copurifying 24 kd protein (216, 219). It is possible that this, rather than the larger component, contains the activity they are measuring. (*d*) The

characteristics of the monoclonal antibodies that are essential reagents in their recent studies have not been published. For example, there has been no description of the cellular proteins with which these antibodies react.

The work of Mazurek et al raises the question of the role of ATP in the opening of the calcium channel (see above), since reconstitution of active channels with purified preparations of receptors and cromolyn-binding protein has been reported by these workers (220). Similarly, it is at present difficult to integrate their findings with those of Ishizaka and her coworkers on the essential role of a serine protease, methyltransferases, and activation of adenylate cyclase in receptor-mediated influx of Ca^{2+} (see above).

Protein kinase activity Phosphorylation and dephosphorylation of proteins are widely used mechanisms by which cells regulate their functions, and several instances in which these mechanisms may control the function of plasma membrane receptors are currently under intensive investigation (221–223). In some of these, the membrane protein is itself a kinase, as well as one of the substrates for that activity (221, 222). Because no biochemical function can yet be assigned to the receptor for IgE, the possible role of phosphorylation in regulating its function can only be explored indirectly. Here we consider three questions: (*a*) Can one or more of the subunits of the receptor serve as a substrate for protein kinases? (*b*) If so, are there changes in the extent (or type) of phosphorylation upon aggregating the receptors, and is this a primary or secondary event? (*c*) Is the receptor itself a kinase?

THE RECEPTOR AS A SUBSTRATE FOR KINASES Hempstead et al (43, 44) observed incorporation of $^{32}P_i$ into phosphothreonine and phosphoserine on the α chain of receptors for IgE on rat mast cells. This finding on the intact mast cells is intriguing because it implies that the α chain may be a transmembrane protein (cf Figure 1). So far, the evidence that α does not penetrate the membrane has been based exclusively on negative results (above) which by their nature are much less convincing. Intact RBL cells incorporate ^{32}P into phosphoserine in the β chains and into undetermined sites in the γ chains, but not into the α chains (41, 42). Using membranes from RBL cells incubated with ^{32}P-ATP, we have observed labeled phosphoserine and phosphothreonine in α chains, and phosphoserine and phosphotyrosine in the β and γ chains (R. Quarto, H. Metzger, unpublished observations). Teshima et al (224) reported incorporation of counts into phosphoserine in a 36 kd protein that coprecipitated with the receptor for IgE when membranes of RBL cells were incubated with ^{32}P-ATP. This kinase activity had the properties of protein kinase C (224, 225). There are insufficient data in that report to determine whether the 36 kd component represents the β chain of the receptor. Finally, we have observed Mn^{2+}-dependent kinase activity in preparations of solubilized receptors that

actively incorporate counts into phosphotyrosine on both the β and γ chains (but not into the α chain or IgE) of solubilized IgE-$\alpha\beta\gamma_2$ complexes (below). Thus, each of the subunits of the receptor for IgE can serve as a substrate for one or more protein kinases.

CHANGES IN AGGREGATED RECEPTORS

Mast cells Hempstead et al reported enhanced phosphorylation of the α chains of the receptor for IgE on mast cells when the latter were reacted with antigen (44). No changes were observed in the regions of the gel where the β and γ chains would have been located.

There are several aspects of this study that make interpretation difficult: (*a*) The purification procedure used in their study (44, 52) would reduce the yields of just those receptors in which one is most interested, i.e. those that had bound IgE on the intact cells and that had been aggregated with antigen. We are not convinced by the evidence that such losses were minimal (44). Furthermore, the purification procedure employed leads to virtually complete loss of the β and γ chains in our hands (30). (*b*) No estimate of the extent of modification was possible so that the fraction of the receptors that were modified is unknown. (*c*) The changes were similar to those observed when the cells were triggered with the ionophore A23187 (43). Since it is unlikely that this drug has any direct effect on the receptors, it suggests that the changes observed in the α chains in both studies (43, 44) may have been secondary ones, e.g. induced by the rise in intracellular Ca^{2+} (226). That RBL cells show no change in the phosphorylation of α chains upon triggering (see below) even though their receptor-mediated secretion in many ways resembles that of normal mast cells, also suggests that the modification of α is not a critical event for activation.

RBL cells Changes in the phosphorylation of the β and γ chains of the receptors from stimulated RBL cells were described by Perez-Montfort et al (42). They used cells that had biosynthetically incorporated ^{3}H-leucine so that the yields of triggered and untriggered receptors could be assessed (they were equivalent) and the change in phosphorylation per unit peptide could be determined. The principal findings were: (*a*) Receptors from antigen-triggered cells showed an average increase in phosphorylation in β of about 45%, whereas the γ chains showed an average decrease of about 35%. In these experiments, the changes in the unaggregated and aggregated receptors from the antigen-treated cells were equivalent. (*b*) The fraction of the receptors that incorporated ^{32}P was small—less than 10% and perhaps much less. This could be assessed because the phosphorylated chains had a slightly reduced mobility on polyacrylamide gels. Neither that study nor a previous one (41) showed evidence of phosphorylation of α chains.

Thus, neither the results of Hempstead et al nor ours provide evidence

that phosphorylation or dephosphorylation of the receptor is a primary event in the early steps initiated by aggregation of the receptors.

INTRINSIC KINASE ACTIVITY OF THE RECEPTOR We recently observed that when receptors that had been purified by the technique we regularly employ (see above) were incubated with ^{32}P-ATP, substantial kinase activity was present (R. Quarto, H. Metzger, unpublished observations). This activity has the properties of a tyrosine kinase and substantial incorporation of phosphorous into tyrosine is observed on both the β and γ chains. When casein is present also, it too is modified but, on a molar basis, much less efficiently than the β and γ chains. If prior to elution of the receptors from the affinity column the latter is briefly "stripped" with micellar detergent (using the same protocol we employ to make the α chain of the receptor susceptible to iodination—see above), activity is found in the detergent wash. These findings suggest that the observed activity may not be due to the receptor itself. To what extent it is specifically associated with the receptor is currently being examined.

Receptor-Induced Inactivation

PRINCIPAL OBSERVATIONS When receptors on mast cells or basophils are aggregated under nonpermissive conditions, such as in the absence of extracellular Ca^{2+}, the cells not only fail to secrete but may become inactivated. That is, restoring permissive conditions—in this case adding back Ca^{2+}—does not lead to degranulation. This inactivation is referred to as desensitization. In some cases the inactivation is specific. For example, using the protocol just cited, if relatively few receptors were initially aggregated in the absence of Ca^{2+}, only these will be incapable of initiating release, whereas others subsequently aggregated after the restoration of mM Ca^{2+} can induce release from the same cells (227, 228). If larger numbers of receptors are aggregated under the nonpermissive conditions, desensitization becomes nonspecific, and it is then no longer possible to initiate any receptor-mediated secretion (227, 228). Studies on RBL cells have given somewhat different results. In this case desensitization only becomes prominent if minimal aggregation of receptors is employed (204, 229), although here too it is likely that the inactivation only involved those receptors aggregated in the absence of Ca^{2+}. The process of inactivation has the following properties: (*a*) It is initiated by aggregation of the receptors, and as with the activation process, dimers of IgE are sufficient but larger oligomers are more effective (96). (*b*) It appears to involve steps prior to the rise in intracellular Ca^{2+}. Thus, the receptor-mediated increase in cellular uptake of $^{45}Ca^{2+}$ is ablated in desensitized cells (230), but if the intracellular Ca^{2+} is raised directly with ionophores, normal secretion is

observed (231). (*c*) The process is an active one and shows interesting parallels to the process that initiates secretion. Thus, inactivation is not observed if the desensitization is conducted at low temperature (232). Furthermore, Kazimierczak et al (138) observed that within a narrow range of doses, DFP when added during aggregation of receptors under nonpermissive conditions could interfere with desensitization as assessed by following secretion from human basophils. Ishizaka et al (137) reported analogous studies on mouse mast cells and showed that the inhibition of desensitization was observable by examining uptake of $^{45}Ca^{2+}$ as well as secretion. The desensitization was not only blocked by very low doses of DFP but also by inhibitors of methyltransferases (137). (*d*) The process of desensitization appears to be a normal accompaniment of the activation process. For example, it occurs even under permissive conditions if the aggregating stimulus is minimal (233 and references therein). Furthermore, in the study by Kazimierczak et al noted above, DFP actually enhanced secretion under permissive conditions if added at appropriate doses (138).

MECHANISM The mechanism by which aggregation of receptors initiates desensitization is uncertain. One particularly simple explanation does not seem adequate. Thus, it might be supposed that during the desensitization phase of the experimental protocols described above, the receptors were shed, degraded, or internalized (234) and were, therefore, not available to be aggregated upon subsequent exposure to the appropriate cross-linking reagents. The observation of MacGlashan et al (235) that *no* internalization occurred with human basophils in the absence of Ca^{2+} could not be substantiated at least with RBL cells (109, 133). However, all studies on this aspect suggest that more than enough IgE-receptor complexes remain on the surface of the desensitized cells to stimulate release.

Thus, the data support the proposal that secretion results from a (positive) balance between activation and inactivation (137, 138, 236). They further suggest that the inactivation occurs as an early event involving reactions similar to those that initiate secretion.

CONCLUDING REMARKS

In our introductory comments, we noted that the receptor for IgE is one of a class of proteins whose interaction with the Fc domains of immunoglobulins transforms the reaction of antibody with antigen into a physiologically important event. In common with other such systems the initiating signal is dependent upon aggregation of the antibody (237, 238).

The proximal component that "senses" the aggregation of the IgE antibody—the receptor—is understood only in broad outline. It is clear

that the α chain binds the IgE, but we know nothing yet about what the β and γ subunits do. Information about the more distal components is based largely on phenomenological data. The molecular nature of these components and how they interact with the receptor and with each other must now be defined. The literature is replete with elaborate flow diagrams—sometimes several in a single paper—that illustrate putative events taking place in the plasma membrane. What is missing is even a single report of a preparation of membranes that demonstrates a meaningful level of any activity initiated by aggregation of the receptor. We are convinced that the goal of developing such preparations deserves highest priority by those wishing to understand the early events mediated by the receptor for IgE.

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CONTENTS

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MURINE MAJOR HISTOCOMPATIBILITY COMPLEX CLASS-I MUTANTS: Molecular Analysis and Structure-Function Implications

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INTRODUCTION

A span of 50 years separates us from the original description of the genetic region termed *H-2*, which was found to control transplant rejection in mice (1, 2). The transplantation genes are now known to be part of the major histocompatibility complex (*MHC*), a multigene cluster located on chromosome 17. At present, at least three major gene families—classes I, II, and III—occupy the *H-2* region. The class-I genes encode the classical transplantation antigens, *H-2K*[1], *H-2D*, and *H-2L* (called *K*, *D*, and *L*), and the differentiation markers, *Qa* and *TL*. Class-II genes encode *Ia* products, involved in antigen presentation to T cells, and the class-III genes encode certain components of complement (see 3).

The *H-2 K*, *D*, and *L* genes each determine a 45,000 M_r glycoprotein (the *MHC* heavy chain) that is associated noncovalently with an 11,600 M_r light-chain subunit (β_2 microglobulin). These two entities, existing as a heterodimeric unit in a 1:1 relationship, comprise the classical *H-2* transplantation antigen (4, 5) (see Figure 1). The heavy chain that anchors

[1] Genetic designations for the *H-2* locus depict the gene in capital letters and the particular haplotype as lower case superscripts, e.g. the *K* gene of the *b* haplotype is *H-2*^{b}.

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the complex in the cell membrane is divided into three extracellular domains (designated α1, α2, and α3), a hydrophobic transmembrane region, and a cytoplasmic region. The domain structure is consistent with class-I gene, exon-intron organization (6).

While historically the *K*-, *D*-, and *L*-gene products were defined as targets in allogeneic homograft rejection, they are now known to play a crucial role in enabling the immune system to recognize and react against cells bearing foreign antigens. One of the key features of the class-I transplantation antigens, thought to play a role in their function, is the extraordinary diversity in primary structure. Studies directed towards understanding the structure-function relationships are hampered by this variability among alleles since subtle, functionally crucial, amino acid alterations are not conspicuous against a background of 15–20% amino acid diversity (5, 7). The spontaneous, in vivo *MHC* mutants have provided a crucial model system for these analyses. The mutant mice, detected by skin graft incompatibility with individuals of the parental haplotype (8, 9, 10), have alterations in their *K*, *D*, or *L*, class-I molecules (11). Further, the nature of alterations in the K^b mutants suggest that they are derived from the genetic interaction of the K^b gene with other class-I genes. Thus, in addition to information necessary for understanding structure-function relationships, the analysis of *MHC* mutants has provided a model for the study of the underlying mechanism of gene diversification.

In this review we focus on mutants of the *H-2K*^{b} gene. We describe the present knowledge of the structure of *K* genes and products from a number of these mutant strains and attempt to relate biochemical changes to altered reactivity in various immunological test systems. We also describe and

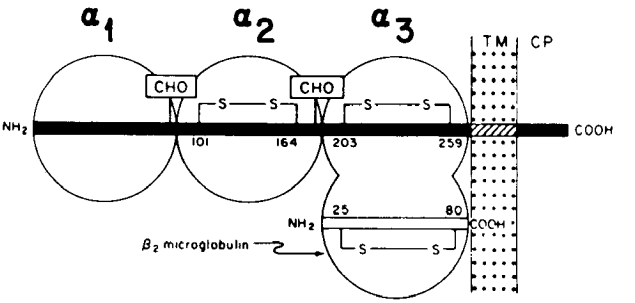

Figure 1 Schematic representation of the *H-2K*^{b} glycoprotein. The postulated three subregions, α1, α2, and α3, comprise the extracellular portion of *H-2K*^{b}, each domain consisting of approximately 90 amino acid residues. The disulfide bridges (SS) are depicted, as are sites of glycosylation (CHO). β_2 microglobulin is shown bound to the third domain, α3. The transmembrane region (TM) is postulated to span the lipid bilayer of the cellular membrane and is followed by the intracytoplasmic carboxy-terminus (CP).

document evidence for a genetic mechanism that plays a role in the diversification of this gene system. Additional model systems designed to produce *MHC* mutants for the purpose of analysis of *MHC* structure-function relationships are also described. Discussion of studies in the homologous human system, human leucocyte antigen (*HLA*), are included where pertinent. The reader is referred to previous reviews for further information on genes and products of the *H-2* system and mutants (3–5, 10–15), and the *HLA* system and variants (16, 17).

IN VIVO CLASS-I MUTANTS

Structural Analysis of K^b *Mutations*

The sequencing of the mutant K^b molecules was undertaken in order to relate altered histocompatibility with precise amino acid changes in class-I molecules. The results of tryptic peptide mapping and amino acid sequencing studies of the parent and mutant K^b molecules (4, 11, 18–21) have recently been confirmed and extended by DNA and RNA sequencing techniques (22–28). The elucidation of the primary nucleotide and deduced amino acid sequences of the mutant molecules, in addition to cloning and restriction enzyme analysis, have provided evidence that the mutant genes are alleles of the *K* locus (19, 25, 29, 30), rather than a series of closely linked *K*-like genes subject to alternative expression.

The results of the above studies are summarized in Table 1, and an amino acid substitution profile of the K^b mutations is diagrammed in Figure 2. When evaluated collectively, the K^b mutations have a number of intriguing properties (for an earlier description of the characteristics of the K^b mutants, see 11 and 19). These properties are:

1. All mutations have occurred in the two amino terminal domains, α1 and α2. This finding supports the idea that the amino acids that contribute to the polymorphic determinants of transplantation antigens are located in this portion of the molecule (5, 11, 31). It must, however, be pointed out that the detection of altered amino acids in mutant K^b molecules is biased by the selection procedure, i.e. skin graft rejection. Mutations at other positions may have occurred, but because they would not have resulted in histoincompatibility, they would not have been detected.
2. Most mutant K^b molecules contain multiple amino acid substitutions. K^{bm10} contains 4 substituted amino acids (28), K^{bm1} contains 3 (23, 25), and K^{bm8} (32) has at least 3 alterations. The only mutant molecules with single amino acid substitutions are K^{bm5} and K^{bm16}, which have been found by amino acid sequencing to contain a Tyr-to-Phe change at position 116 (20).

Table 1 Molecular characteristics of K^b mutations and donor genes

Mutant	Position of altered amino acids[1]	Altered nucleotides/ consecutive nucleotides[2]	Donor gene	Length of identity encompassing mutation[3]	References
K^{bm1}	152 Glu → Ala 155 Arg → Tyr 156 Leu → Tyr	7/13	Q10	50	19, 23, 25, 43
K^{bm8}	22 Tyr → Phe 23 Met → Ile 24 Glu → Ser	—[4]	—	—	19, 32
K^{bm5}	116 Tyr → Phe	—	—	—	20
K^{bm16}	116 Tyr → Phe	—	—	—	20
K^{bm6}	116 Tyr → Phe 121 Cys → Arg	2/15	Q4	95	21, 26
K^{bm7}	116 Tyr → Phe 121 Cys → Arg	—	—	—	21
K^{bm9}	116 Tyr → Phe 121 Cys → Arg	2/15	Q4	95	21, 27
K^{bm3}	77 Asp → Ser 89 Lys → Ala	4/38	—	—	11, 28, 39
K^{bm11}	77 Asp → Ser 80 Thr → Asn	3/11	D^b	45	11, 28
K^{bm23}	75 Arg → His 77 Asp → Ser	4/7	Q10	52	44
K^{bm4}	173 Lys → Glu 174 Asn → Leu	3/5	K1[4] Q4 Q10	— 26 21	28
K^{bm10}	163 Thr → Ala 165 Val → Met 173 Lys → Glu 174 Asn → Leu	5/35	K1	—	10, 28

[1] Based on available sequence data

3. Multiple nucleotide and resultant amino acid substitutions have always occurred in a cluster. For example, the K^{bm1} gene contains 7 altered nucleotides in a span of 13 (23, 25). The most extended clusters are found in K^{bm3} and K^{bm10} genes, which are altered in 4 of 38 and 5 of 35 nucleotides, respectively (28). Furthermore, alterations in the primary sequence are limited to a single cluster per molecule (11, 25, 26). This has also been found to be the case for *K*-gene mutants of the *k* and *f* haplotypes (33, 34).
4. Identical amino acid substitutions have been detected in the altered K^b molecules of several independently arising mutant mice. The most striking example of this is the "bg series" of mutants—(bm5, bm6, bm7, bm9, bm16, bm17 and bm20) (35). The "bg series" of mutants are histocompatible with one another, although they reject parental $H\text{-}2^b$ skin grafts (35). Amino acid sequencing studies have determined that K^{bm5}, K^{bm6}, K^{bm7}, K^{bm9}, and K^{bm16} gene products have a Tyr-to-Phe substitution at position 116 (20, 21). In addition, K^{bm6}, K^{bm7} and K^{bm9} products have a second substitution, Cys-to-Arg, at position 121 (21). Further analyses have demonstrated that K^{bm6} and K^{bm9} are identical

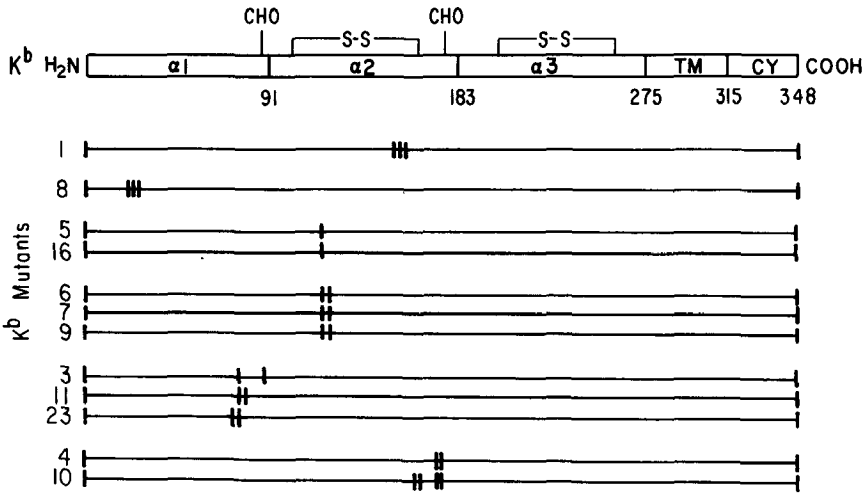

Figure 2 Amino acid substitution profile of the K^b mutants. The K^b molecule is represented as an empty rectangle; K^b mutants are depicted as solid lines. Domain structure of K^b is according to exon-intron organization as described by Weiss et al (22). The three extracellular domains are α1, α2 and α3; the transmembrane domain is abbreviated, TM; three cytoplasmic exons are presented in a single domain, CY. Amino acid residues are numbered according to Nathenson et al (4); the numbering of amino acids at domain junctions is according to Weiss et al (22). Disulfide bridges are represented as S-S and carbohydrate moieties are represented by CHO. Vertical dashes represent known amino acid alterations. Where sequence data are incomplete additional amino acid substitutions may be present.

on the nucleotide level, indicating that the same mutation has occurred at least twice (26, 27).

5. Many amino acid substitutions have involved multiple nucleotide alterations per codon. Of the collective 13 substituted amino acids in all of the mutant K^b alleles analyzed, 7 amino acid substitutions involve 2 or more nucleotides per codon (Table 1). In particular, of the three altered codons of the K^{bm1} gene, two have a double nucleotide substitution and one has three substitutions (23, 25).
6. The substituted nucleotides and replaced amino acids present in mutant K^b genes are found, at homologous positions, in other class-I genes of various haplotypes. This observation was originally made on the amino acid level by Evans et al (36) and Pease et al (19).

Genetic Recombination[2] *Generates the* K^b *Mutants*

The features of the K^b mutants suggest that they are not the result of simple, random point mutations, but rather, are due to a more complex genetic process. This led to the hypothesis that genetic interaction between class-I genes may be the driving force behind the production of the K^b mutants as well as the generation of diversity in *H-2* antigens (19, 22, 31, 36–42). In fact, this hypothesis was used as a basis for the identification of additional altered amino acids in several K^b mutant molecules (19).

DONOR GENES An extension of the recombination hypothesis is the prediction that donor sequences, capable of interacting with the K^b gene, would be present in the genome of the mouse strain in which the mutation arose (25). Synthetic oligonucleotides were constructed to mutant DNA sequences and used as probes to identify donor genes from the $H\text{-}2^b$ haplotype. The potential of the donor genes to provide the mutant sequence was confirmed by DNA sequencing (26–28, 43, 44). Thus, the prediction of the existence of donor genes was borne out by the identification of class-I genes in the $H\text{-}2^b$ haplotype that contain nucleotide sequences identical to those substituted into mutant K^b genes.

Notably, class-I genes of the *K*, *D*, and *Qa* regions of the *H-2* complex are capable of interacting as donors with the K^b gene (Table 1, Figure 3). The *Q10* gene contains sequences identical to the substitutions in the K^{bm1} and K^{bm23} mutant genes (43, 44). Genetic interactions between the *Q4* and K^b genes have been demonstrated to be responsible for the generation of two

[2] While the term *gene conversion* has been used by a number of groups to describe the findings of genetic transfer between class-I genes, proof of such a mechanism, as defined in lower eukaryotes, is not available. Thus, we will utilize the term *recombination* in its broadest sense, referring to the rearrangement of DNA sequences by the reciprocal or nonreciprocal mechanisms of unequal crossing over, gene conversion and postmeiotic or mitotic segregation.

independent, identical mutant genes, K^{bm6} and K^{bm9} (26, 27). The *K1*, *Q10* and *Q4* genes are identical with K^{bm4} at positions 173 and 174 and might be potential donor genes for the K^{bm4} mutation (28). The K*1* gene, however, is the only possible donor for the related K^{bm10} gene (28). The D^b gene is the donor for the K^{bm11} gene (28).

No gene of the *TL* region has as yet been found to contain a donor sequence. Perhaps *TL* genes lack sufficient identity with K^b to initiate recombination (3, 45, 46). Alternatively, *TL* sequences substituted in the K^b gene may not be detected because selection at the protein level may eliminate *K* molecules that have been altered with *TL* sequences (3, 23, 43, 47).

The amount of genetic transfer to K^b from other class-I donor genes ranges from a potential minimum of 5 nucleotides for the K^{bm4} gene to a potential maximum of 95 nucleotides for the K^{bm6} and K^{bm9} genes (Table 1). *Q4*, the donor gene for the K^{bm6} and K^{bm9} genes and possibly for an additional 6 related "bg series" genes, shares the longest length of identity with K^b (95 nucleotides) surrounding the substitutions at the codons corresponding to amino acid positions 116 and 121. After a silent single base mismatch in the codon for amino acid 108, K^b and *Q4* share an additional 64 bases of identity (26). This relatively great extent of identity between *Q4* and K^b may be responsible for increased recombination between the two genes and the subsequent high frequency of "bg series" mutations (26).

The amount of genetic information transferred between class-I genes in the generation of the K^b mutations is relatively small compared to other mammalian gene systems that often indicate genetic transfers of several hundred nucleotides (42, 48–50). The limited alterations observed in the mutant K^b genes thus far examined have involved only exons (with the

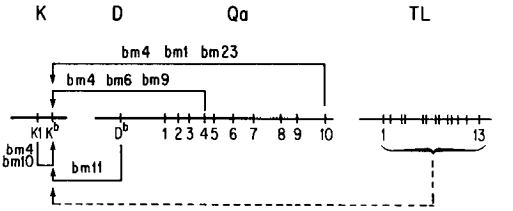

Figure 3 Generation of K^b mutants by the transfer of DNA from donor genes to K^b. Uppermost capital letters represent the *K*, *D*, *Qa*, and *TL* regions of the *MHC*. *K* region genes, *K1* and K^b, are indicated; *Qa* region genes are numbered 1–10, and *TL* region genes are numbered 1–13 according to Weiss et al (29). Mutants are indicated next to arrows connecting donor genes and K^b. The donor gene that generated the bm4 mutant might have been *Q4*, *Q10*, or *K1*, based on limited sequence data.

possible exception of K^{bm3}), whereas recombination in other gene systems often involved both exons and introns.

A summary of class-I donor gene interactions with the K^b gene is presented in Figure 3. This figure depicts the transfer of genetic information from donor genes to K^b. It is not known whether the K^b gene is capable of donating genetic sequences to other class-I genes. Due to the selection procedure for mutant mice, the transfer of K^b sequences into a recipient gene (e.g. *Q10*) may not be detectable. The examination of *D*-region mutants (bm13 and bm14), selected by histoincompatibility (51), may provide the opportunity to determine whether K^b can serve as a donor gene for other class-I genes.

Several features associated with the K^b mutants are also evident in naturally occurring variants of *HLA* class-I molecules. For example, the *HLA-B27.2* variant of *HLA-B27* contains substitutions at amino acid positions 77, 80, and 81 (52), a region altered in K^{bm3}, K^{bm11} and K^{bm23}. The *E1-A3* variant of *HLA-A3* has alterations at amino acid positions 152 and 156 (53), which resemble the positions of change in K^{bm1}. The *CF* variant of *HLA-B7* has an unidentified change at position 116 (54), identical to the position of change in K^{bm5} and K^{bm16} molecules. Not only are the substituted positions very similar to those of the K^b mutant molecules, but the known, substituted amino acid clusters are present, at homologous positions, in other human class-I gene products. Thus, gene interaction apparently plays a major role in the diversification of class-I transplantation genes of both mouse and man.

MITOTIC RECOMBINATION The genetic interactions that lead to the formation of mutant K^b genes probably occur in the germ-line cells of either the male or female mouse, although it has been suggested (55) that there may be a bias toward recombination in the female. Recombination may occur during the mitotic amplification of the germ-line cells or the pairing of chromosomes in meiosis, or following fertilization of the ovum. Since many of the bm mutant mice were detected in the offspring of C57BL/6 × BALB/c ($H\text{-}2^b \times H\text{-}2^d$) matings (11, 35, 55), it has been suggested that the K^b gene may have interacted with a donor gene from either the $H\text{-}2^b$ or $H\text{-}2^d$ haplotype in the fertilized egg (3, 23, 43). Thus, the K^{bm9} mutation may have arisen via zygotic recombination between K^b and either $Q4^b$, K^d, or possibly some other gene in the $H\text{-}2^d$ haplotype. However, genealogical analysis indicates that the K^{bm9} gene was detected in 5 of 19 siblings (35). Sibships with multiple mutant mice have also been identified for bm6 (3/14) and bm23 (4/12) (35, 56). These findings argue against both zygotic and meiotic recombination; only one ovum can result from a single meiotic recombination, and the probability of the identical zygotic recombination occurring as many as five times in a single sibship is nil.

A more plausible explanation for multiple, identical mutants in a single sibship is that one of the parents (the female in the case of bm9 and bm23) was a gonadal mosaic, having germ cells of which a sizeable percentage (up to 33%) carried the same mutant allele (12, 35). On the molecular level, this may be explained by the occurrence of mitotic recombination early in the expansion of the germ line, such that a large fraction of the germ cells contained the mutant allele. Cases in which a single mutant was found in a sibship can be explained by the occurrence of a mitotic recombination late in ontogeny, resulting in a decreased percentage of the mutant allele in the germ-cell pool. Thus, it is possible that all of the K^b mutations occurred in the mitotic amplification of the germ line (27).

Generation of Diversity in Class-I Genes

The genetic homogeneity among members of an inbred mouse strain represents a system in which the evolutionary time clock has been reset to zero. Each recombination event leading to a mutation in K^b is, in effect, a step in the rediversification of the C57BL/6 genome. As substitutions accumulate due to recombination between the K^b gene and the various class-I donor genes, the K^b gene would acquire the characteristics of other class-I genes. The effect of sequential mutations in a single K^b gene is depicted in the hypothetical scheme in Figure 4. In addition to the *Q4*, *Q10*,

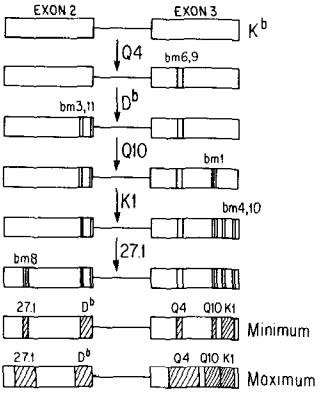

Figure 4 A hypothetical diversification scheme for the K^b gene. Exons 2 and 3 (domains $\alpha 1$ and $\alpha 2$) are depicted as empty rectangles. Vertical lines within the exons represent altered codons resulting from recombination between K^b and donor gene. The D^b gene is depicted as the donor gene for both the K^{bm3} and K^{bm11} mutations as it contains the appropriate sequence at positions 77, 80, and 89. The *27.1* gene of the *H-2*^{d} haplotype is used as a hypothetical donor gene for the K^{bm8} mutation on the basis of amino acid identity. Hatched areas represent the potential minimum (nucleotides within the first to last substitution) and potential maximum (consecutive, identical nucleotides between the mutant K^b gene and the donor gene in the region of the mutation) transfer of DNA from donor gene to K^b.

K1, and D^b donor genes known to interact with K^b, a *Q* gene of the $H\text{-}2^d$ haplotype, 27.1, is included because it contains the same amino acid sequence as the K^{bm8} mutation. The hatched areas in Figure 4, which indicate the maximum extent of recombination between the donor genes and K^b, demonstrate that after several interactions, the second and third exons of K^b are almost 60% substituted with sequences from other class-I genes. Hence, the lack of K-ness and D-ness among transplantation antigens can be explained by this phenomenon: Genetic interactions, as illustrated in Figure 4, have changed K^b into a mosaic of *K*, *D*, *K1*, *Q4*, and *Q10* gene sequences. Thus, the recombination events generating the K^b mutants may be representative of the underlying mechanism involved in the ongoing evolution of class-I genes.

A possible example of diversity created by sequential recombinations may be the spontaneous human class-I variant *HLA-B27.3* which, when detected, had already undergone two distant substitutions, one at amino acid 77 and the other at amino acid 152, positions also affected in the K^b mutant series (57).

Conservation and Diversification of Sequence in Class-I Genes

Class-I genes are distinguished by both diversity and conservation of sequence. Exons 2 and 3 of the *K*, *D*, and *L* genes show extensive allelic diversity, in contrast to the limited diversity exhibited for these exons in alleles of *Qa* and *TL* region loci (45–47, 58–60). Short recombination events, such as those observed in the generation of the K^b mutants, can serve as a mechanism for the diversification of alleles of the *K*, *D*, and *L* loci. The absence of diversity in the alleles of *Qa*- and *TL*-region loci may reflect the inability of these genes to serve as recipients of genetic information in short recombination events (3, 23, 43, 47). Alternatively, alterations in the amino terminal portion of *Qa* and *TL* molecules may be severely restricted by functional constraints and natural selection (3, 23, 43, 47), while such alterations are selected for in *K*, *D*, and *L* alleles.

Sequence conservation exists within the fourth exon (α3 domain) where all class-I genes show extensive homology. Functional constraints imposed by the binding of β_2m to the α3 domain appear to be responsible for the amino acid sequence conservation (61). Furthermore, the homology among α3 domains of class-I molecules extends beyond amino acid sequence into the nucleotide sequence of exon 4. This can be explained by the occurrence of long recombination events that would serve to maintain sequence homology. Therefore, short and long recombination events may work in concert to produce a multigene family with properties of both sequence diversity and conservation.

IN VITRO CLASS-I VARIANTS

Somatic Cell Variants

The analysis of mice bearing spontaneous mutations of the K^b molecule has provided the foundation for an understanding of *MHC* class-I protein structure in relation to function. Studies of these mutant mice offered our initial insight into the regions of the class-I heavy chain involved in allogeneic rejection and *MHC* restriction. Nevertheless, the in vivo mutants are few in number, resulting in a limited appreciation for various functional regions of K^b. Additionally, as previously mentioned, the mutations are often complex nucleotide alterations resulting in clustered, multiple amino acid changes. Thus, from analysis of K^{bm} variants, it has been difficult to define the minimal biochemical change—i.e. mutation in a single amino acid residue—that will give rise to critical functional alterations. This limitation has necessitated the additional approach of generating variants of *H-2* genes in established cell lines.

Passage of tumors from F1 animals in parental strains has been used to obtain *H-2* haplotype loss variants (62). Selection in vitro, with anti-*H-2* alloantisera, identified stable leukemia cell variants that had lost cell-surface expression either of an entire haplotype or of a single *MHC* class-I antigen (63–65). The use of monoclonal antibodies to select somatic variants with more defined alterations in a specific *H-2* antigen was first performed by Rajan (66). This latter study utilized the property of monoclonal antibodies to react with selected regions of class-I heavy chains (67). By using a single monoclonal antibody as a selecting reagent, one could thus identify variants that had lost a single serologically defined antigenic epitope. With this negative selection approach, it was possible to identify mutagen-induced *H-2* variants with altered antigenic properties recognized by both antibody and T cells (68–70). An additional approach employing positive selection has been used successfully to identify $H\text{-}2K^k$ variants (71).

A series of chemically mutagenized K^b variants with a wide range of serological phenotypes (72) have been isolated. Flow cytofluorometric analysis of these variants has been performed with seven monoclonal antibodies recognizing either the $\alpha 1$ or $\alpha 2$ domain. Using this array of monoclonal antibodies, two classes of K^b variants have been identified. These include (*a*) structural variants with altered phenotypic expression of the K^b molecule, evidenced by the loss of reactivity with certain monoclonal antibodies, but retention of reactivity with others, and (*b*) loss variants with a total loss of expression of K^b molecules at the plasma membrane surface, revealed by nonreactivity with a pool of all seven monoclonal antibodies. Similar types of somatic changes have been identified in mutagen-induced

HLA-A2-variant cell lines (73). *HLA* variants have also been identified that secrete class-I heavy-chain products (74, 75).

ANALYSIS OF MHC STRUCTURAL VARIANTS Somatic cell variants have been obtained that express the K^b molecule with a variety of antigenic forms. In the majority of cases the monoclonal antibody specificity originally selected against is the only K^b epitope lost. This phenotype characterized by loss of a single epitope was observed in 18 of the 32 independently derived cell lines we have tested to date. This pattern is presented for several different selecting monoclonal antibodies in Table 2A. These mutant cell lines presumably have limited structural alterations that result in minimal antigenic changes. In addition to the variant cell lines with an alteration in the binding pattern of a single monoclonal antibody, a second group of immunoselected variants possess a more complex K^b phenotype. Among the latter group of variants, selection with a monoclonal antibody to a given domain (either α1 or α2) results in the loss of binding of monoclonal antibodies reactive with the same domain. For example, variant 208 (see Table 2B) selected with an α1-domain-reactive antibody, EH144, lost reactivity with monoclonal antibody B8-24-3, also reactive with the α1 domain. This same pattern has been observed for selecting monoclonal

Table 2 Somatic cell variants of K^b with domain-specific monoclonal-antibody loss phenotype

	Anti-K^b monoclonal antibody						
Cell line	EH144 (α1)	B8-24-3 (α1)	K9-136 (α1/α2)	28-13-3 (α2)	K9-178 (α2)	5F1.2.14 (α2)	Y-3[1]
R8[2]	+	+	+	+	+	+	+
			A. Single mAb loss				
R8.246	−	+	+	+	+	+	+
R8.205	+	+	−	+	+	+	+
R8.8	+	+	+	−	+	+	+
R8.332	+	+	+	+	+	−	+
R8.14	+	+	+	+	+	+	−
			B. Multiple mAb loss				
R8.208	−	−	+	+	+	+	+
R8.70	+	+	+	−	−	+	+
R8.335	+	+	+	+	−	−	+

[1] Not mapped.

[2] R8 is a heterozygous (H-2^b × H-2^d) pre-B lymphoblastoid cell line immortalized by Abelson virus transformation. Mutants derived from R8 were obtained by chemical mutagenesis with either ethylmethane sulfonate or ethyl nitrosourea (Geier et al 72).

antibodies to the α2 domain and loss of reactivity with other α2-reactive monoclonal antibodies (Table 2B). Such findings support the idea that antibodies bind to specific domains of the *H-2* molecule, consistent with the results of monoclonal antibody–binding studies on the in vivo mutant K^b molecules (67, 76) and on hybrid *H-2* molecules produced by exon shuffling of *H-2* genes (77–79).

Analysis of *HLA* class-I variants produced by a similar procedure of mutagenesis followed by immunoselection has been performed by Pious et al (73, 80). Selection with an *HLA-A2*-specific monoclonal antibody identified structural mutants from a B-lymphoblastoid cell line which, although nonreactive with the selecting monoclonal antibody, retained reactivity with other *HLA-A2* specific antibodies (73). Structural analysis of three such variants (81, 82) defined residues important for antibody recognition of the *HLA-A2* product. Alterations in the tryptic peptide comprising amino acid residues 98–108 in the α2 domain have been identified for two independently isolated variants (81). Although the mutations appear different, the exact substitutions are not known. A third variant, which fails to react with at least two *HLA-A2*-specific antibodies, has undergone a mutation resulting in the replacement of lysine for glutamic acid at position 161 (82). These results point to the α2 domain for the expression of certain *HLA-A2* antigenic epitopes and specifically to glutamic acid at position 161 in the formation of at least two epitopes recognized by monoclonal antibody.

Recombinant H-2 *Genes*

The advent of gene-cloning procedures and DNA-mediated gene transfer into eukaryotic cells has established an entirely new direction for production and analysis of mutant or hybrid *H-2* products. *MHC* genes have now been cloned from several haplotypes including $H\text{-}2^b$ (24, 29), $H\text{-}2^d$ (30, 36, 83, 84), $H\text{-}2^k$ (85), and $H\text{-}2^q$ (86). Introduction of cloned *H-2* genes into cultured mammalian cells results in the stable expression of allogeneic class-I products on the cell surface (36, 87–92). The recent development of intraembryonic injection of cloned genes has, in addition, produced transgenic mice expressing foreign class-I genes (93). These approaches enable isolated *MHC* products to be studied in different microenvironments, separated from the class-I molecules with which they are usually coordinately expressed.

A number of approaches, including exon-shuffling (77–79, 94–97), deletion mutagenesis (98, 99), and oligonucleotide-directed, site-specific mutagenesis (100, 101), have been utilized in order to construct altered *MHC* genes. Recombinant constructions have been prepared from K^b/D^b

(96), K^b/K^k, K^d/K^k, K^b/K^d (97), and L^d/D^d (77, 94, 95). These genes, when transfected into tissue-culture cells, are expressed as hybrid products. Thus, using this approach it is possible to study the immunobiology of isolated class-I domains, a topic discussed in the following section. The construction of interspecies class-I hybrids (102) and *H-2* class-I/class-II chimeric genes (103) should prove useful for determining the degree of functional relatedness for *MHC* genes derived from different species and neighboring loci.

Regulatory Variants

In addition to the structural variants of *H-2* and *HLA*, immunoselected cell lines also have been identified that no longer express specific *MHC* products on the cell surface. Such lines have proven useful for determining the molecular and structural mechanisms involved in directing a particular class-I protein to the cell surface.

Somatic cell variants have been identified that either do not translate a specific *MHC* product or that synthesize an altered intracellular product (73, 104, 105). Molecular analysis of variants that do not translate a specific heavy chain, described for both *H-2* (105, 106) and *HLA* (107, 108), reveals in some cases an absence of the corresponding class-I messenger RNA. Studies designed to determine the nature of this defect in *HLA* variants have employed somatic cell fusion and have identified *trans*-acting regulatory factors for proper *HLA* class-I gene expression (108). Although the basis for the transcriptional defect described in *H-2* variants is not completely understood, it is expected that a detailed analysis of the region of the genome within or surrounding the altered gene will provide important information concerning the normal expression of *H-2* class-I genes.

Variants that synthesize an altered intracellular molecule provide an opportunity to determine the structural features involved in transport and expression of class-I products. The synthesis of *HLA-A2* heavy chains that associate with β_2m either inefficiently or not all gives rise to very low or undetectable levels of the *HLA-A2* protein on the variant cell surface (73, 104). In contrast, an $H\text{-}2K^b$ variant has been identified that synthesizes an intracellular product and, although not expressed on the cell surface, is found associated with β_2m (109). Normal levels of K^b mRNA have been found in this latter variant, suggesting posttranscriptional, perhaps structural, regulation of $H\text{-}2K^b$ expression. Thus, alterations resulting in the loss of association with β_2m, or alterations in the heavy chain not necessarily correlated with loss of β_2m association, potentially can influence class-I antigen expression. The dm2 mutant mouse, which has sustained a complex mutation in the L^d molecule, revealed by tryptic peptide map analysis,

provides an example of an intracellular localized class-I product occurring in vivo (110).

The collective studies of several laboratories employing gene modification suggest that the cytoplasmic domain (98, 99), α2-domain disulfide linkage (100), and carbohydrate moieties (101) may not play a significant role in transport of L^d class-I heavy chains to the plasma membrane surface. These studies, utilizing different approaches for producing in vitro variants of class-I genes and their products, provide an entirely new direction for identifying the particular features of class-I molecules involved in regulation of their expression.

STRUCTURE-FUNCTION IMPLICATIONS ARISING FROM STUDIES OF CLASS-I MUTANTS

Cellular Recognition of In Vivo Variants

Our present concept of the function of class-I molecules concerns their property of interacting with cytolytic T cells. The mutants have proven to be a valuable resource for obtaining information on the two general functional attributes of the class-I products: (*a*) allogeneic recognition, and (*b*) *H-2* restricted or antigen-associated recognition. Allogeneic recognition refers to the recognition by cytolytic T cells of the *MHC* class-I product leading to the lysis of the target cell. This type of cellular immune recognition is found in graft rejection, a phenomenon that originally allowed the discovery and description of the class-I products (111). *MHC*- or *H-2*-restricted antigen recognition refers to a process whereby foreign antigens are recognized in the context of a particular class-I molecule (112).

The K^b mutants have been studied in a variety of functional assays in which they serve as specific targets for recognition by alloreactive CTLs (113–115), virus-restricted CTLs (116–123), and minor histocompatibility antigen-restricted CTLs (124–126). From this complex array of studies, one can attempt to correlate the structure of the K^b molecule to its recognition by the cellular immune system.

ALLOGENEIC RECOGNITION Studies on CTL alloreactivity by Melief and coworkers (113) and Egorov & Egorov (56) established the relatedness of the bm mutants to parental mice and to each other. Functional relatedness among mutants is consistent with the finding of a similar or identical amino acid sequence of their K^{bm} molecules. For instance, bm3, bm11, and bm23 demonstrate similar patterns of reciprocal CTL alloreactivity, and their K^b molecules share a common change (amino acid position 77, Asp to Ser) (11, 28, 44). The bm6 and bm9 mutants, as another example, exhibit identical CTL alloreactivity. The basis for their similarity in function is now evident

from sequence analysis that demonstrated complete identity of their K^b genes (26, 27). The changes in the bm1, bm8, and bm10 K molecules generate unique alloreactive epitopes that are not shared among themselves or with other mutants. These studies support the concept that there exist mutant-specific as well as shared functional CTL determinants.

In a series of studies using cloned alloreactive CTL lines, rather than primary cultures, Sherman (114, 115) has confirmed and extended the studies described above using a number of mutant/parent combinations. In one group of parent anti-bm11 clones, it was found that, in addition to reacting with the stimulator-bm11 cells and the related bm3-mutant cells, a number of CTLs cross-reacted on other mutant target cells that had alterations in stretches of the K^b molecule different from K^{bm3} and K^{bm11}. These target K^b molecules had changes in either the $\alpha 1$ or $\alpha 2$ domain. In concert with the observations of Melief et al (113) on cross-reactivity of primary mutant/parent CTL, such data suggested that CTLs recognize conformational determinants produced by interaction of residues located at different sites on the heavy-chain polypeptide. Additional support for the concept that CTLs do not recognize sites consisting of linear amino acids in a single domain comes from the work of de Waal et al (127), in which CTLs generated to K^{bm1} were not reactive with L^d although K^{bm1} and L^d share the stretch of amino acids from 146–161, containing the mutant site 152–156.

MHC RESTRICTION The functional characteristics of the K^b mutants have also been evaluated in virus restriction assays. A number of viruses appear to be recognized by CTL in the context of class-I molecules, and Table 3 lists an extensive review of this series of studies utilizing either virus-infected mutant or parental cells as targets for K^b-restricted, virus-specific CTLs. The bm1 mutant, which has changes in K^b at amino acid positions 152, 155, and 156, is in all cases the most different from other mutants and from the parental type. This mutant appears to define a crucial region for CTL recognition. The basis for this effect on CTL/*MHC* recognition is not completely understood but must certainly relate to the nature and position of the amino acid changes.

The bm 5, 16, 6, 7, and 9 mutants ("bg series") share a similar spectrum of reactivity for a number of viral systems, fitting with structural similarities of their K^b molecules. These mutants appear to be the most closely related to the parental B6 mouse in these analyses. The bm3 and bm11 mutants exhibit similar, but not identical, profiles as viral restriction elements, again consistent with their partially shared structural changes. Other *MHC*-restriction assay systems, i.e. association with minor H antigens, also suggest a relatedness between bm3 and bm11 (125, 126), as do patterns of alloreactivity on haptenated mutant target cells (128, 129). These data

Table 3 Effect of $H\text{-}2^{bm}$ mutation on recognition by virus-specific K^b-restricted cytotoxic effector cells

	Virus-infected mutant target cells								
Mutant	bm8	bm11	bm3	bm5, 16	bm6, 7, 9, 17	bm1	bm10	bm4	
Altered residues[1]	22, 23, 24	77, 80	77, 89	116	116, 121	152, 155, 156	163, 165, 173, 174	173, 174	
Affected domain	α1	α1	α1	α2	α2	α2	α2	α2	References
Effector									
αK^b Ectromelia	++[2]			±	++	−			116
αK^b LCMV	+			++	++	−			116
	−	++		+++		−	+		123
								+++	117
αK^b SV40	++	++	+++	+++	++	−	++		119
			++					+	118
αK^b Sendai	±	−	±		++	−			127
	+	−	+	+	+	−		+	121
αK^b Vaccinia								+++	117
			±	++	+	−	+		139
αK^b Influenza						−			140
αK^b VSV	+	−	−	+	+	−			121
αK^b Moloney	−	++	++	++	++	−			120

[1] Positions of known amino acid substitutions.

[2] (−) Indicates 0–10% of the cytolysis in the positive control in the reference cited; (±) indicates 10–25%; (+) indicates 25–50%; (++) indicates 50–75%; (+++) indicates 75–100%.

suggest that the structural features of the mutant K^b molecule determine its recognition by CTL, to a large degree independent of the foreign antigen functional system used.

An overall conclusion from these studies has been that small alterations play a crucial role in the recognition by the CTL for *MHC*-plus-foreign-antigen; in addition, conformational determinants must be implicated to explain loss of CTL recognition of different K^b molecules in which there are changes in sites linearly distant from each other. That is, amino acid changes in either the α1 domain or the α2 domain can alter the recognition by parental K^b-restricted virus-specific CTLs. Such is the case for Sendai virus-restricted CTL that no longer recognize virus-infected bm1 (α2) or bm11 (α1) (Table 3), and also for Moloney-virus-restricted CTL that no longer recognize virus-infected bm1 (α2) or bm8 (α1).

At least two components are involved in the recognition by CTL of foreign antigen in the context of class-I molecules. Thus, the loss of recognition of virus in the context of mutant K^b may be due to the inability of the virus or viral products to associate with the mutant K^b molecule; or alternatively, it may be due to an alteration in the site on the *MHC* class-I molecule to which the T-cell receptor binds. At present we have little information on these different aspects of the recognition process.

While we have primarily described mutants of K^b, mutations in D^b also occur. Two D^b mutants, bm13 and bm14, have been isolated (51) that are histoincompatible with parental mice as well as with each other. These mutants also have marked alterations in virus restriction properties. Thus, in Moloney virus infection, bm13 and bm14 lose the D^b-restricted response. The bm13 mutant transfers restriction to K^b, while bm14 is a total nonresponder (122).

Analysis of In Vitro Variants

As previously mentioned, monoclonal antibody–selected *H-2* and *HLA* somatic-cell variants have been isolated. The conclusion has emerged from analysis of these variants that antibody and CTL recognize different determinants on the class-I heavy chain (70, 72). In a series of studies using cloned CTL, a number of the variants described in Table 2A and B were studied, and in results similar to those obtained with in vivo mutants (115), different CTL clones showed a complicated pattern of cross-reactivity on the in vitro selected mutants (70). For example, bm10 anti-K^b CTL clones would be expected to recognize sites within the α2 domain of K^b molecules dependent upon a polypeptide stretch that includes residues from 163 to 174. However, a majority of these CTL clones lost recognition of mutants with putative changes in the α1 domain (since the mutants were selected with an anti-α1 domain monoclonal antibody). Therefore, much like the conclu-

sions drawn from the in vivo variants, CTLs appear to recognize determinants present on in vitro variants formed by interaction of the α1 and α2 domains (70).

The immune recognition of hybrid *H-2* gene products produced by exon exchange between different class-I genes (see Recombinant *H-2* Genes) has also been analyzed. Again, the alloreactive CTL determinants for these hybrid genes were shown to be located in the α1 and α2 domains (78, 79, 94, 95, 96). The analyses further suggested that interaction between α1 and α2 gives rise to conformational sites recognized by both allogeneic (79, 96) and virus-specific CTL (96, 97) and that α3 does not significantly influence these sites (96, 97). Thus, the analysis of in vitro variants produced both by chemical mutagenesis and genetic engineering confirms and extends the results obtained with the naturally occurring mutants.

Deletion mutagenesis and exon-shuffling have been utilized to identify the functional role of the class-I cytoplasmic domain in CTL recognition of *MHC* products. Excision of this region from cloned L^d genes yielded disparate results in T cell–mediated killing of vesicular stomatitis virus–infected cells. Zuniga et al (99) demonstrated that lysis was independent of the cytoplasmic domain of L^d whereas Murre et al (98) suggested that the truncated L^d molecule was recognized less efficiently than the intact molecule. Analysis of CTL directed against either lymphocytic choriomeningitis virus (99) or influenza virus (98) in the context of *MHC* has shown lack of a requirement for the L^d cytoplasmic domain. Similarly, influenza-A-virus recognition by K^b restricted CTL is independent of the phenotype of the intracellular C-terminus (97). By consensus, therefore, it appears that the cytoplasmic domain does not play a crucial role in *MHC*-restricted T cell–mediated cytotoxicity.

Antibody Recognition of K^b *Variants*

Using a series of monoclonal antibodies raised for a number of different allogeneic combinations by several laboratories, studies on K^b mutant and parent molecules have revealed relevant features of the antigenic determinants on *H-2*K^b with which these antibodies interact. It is striking that of 15 monoclonal antibodies against K^b derived from various alloimmunizations (56, 67, 76, 130), 10 were found to react specifically with sites located in either the α1 or α2 domain. Of these, 5 were localized further to epitopes influenced by alterations in amino acid positions 75, 77, or 89, since monoclonal antibodies EH-144 (T. V. Rajan, unpublished), B8-24-3 (131), 20-8-4 (132), K10-56 (67), and Y-25 (133) were weakly bound or unreactive with cells from the mutants bm3 and 23 (Table 4). The bm11 mutant cells, while unreactive with Y-25 retained reactivity with other monoclonal antibodies of this series. Significantly, determinants detected by five other

Table 4 Properties of mutants altered in the α1 recognition region (70–90)

	Amino acid sequence																				mAb profile[1]					
	70					75					80						85				90						
	N	E	Q	S	F	R	V	D	L	R	T	L	L	G	Y	Y	N	Q	S	K	G	B8-24-3	EH-144	20-8-4	K9-136	K10-56	Y-25
		–				+		–		+							*			+						
K^b	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+[2]	+	+	+	+	+
bm3	—	—	—	—	—	—	—	S	—	—	—	—	—	—	—	—	—	—	—	—	A	–	–	–	–	–	–
bm11	—	—	—	—	—	—	—	S	—	—	N	—	—	—	—	—	—	—	—	—	—	+	.[3]	+	+	+	–
bm23	—	—	—	—	—	H	—	S	—	—	—	—	—	—	—	—	—	—	—	—	—	–	.	–	.	.	.

* Carbohydrate moiety.
[1] Data are compilation of references 67, 76, 130, 56.
[2] Key: Reactivity in different studies: (+) greater than 25%; (±) 10–25%; (–) 0–10%.
[3] Not determined.

Table 5 Properties of mutants altered in the α2 recognition region (150–180)

	Amino acid sequence						mAb profile[1]					
	150	155	160	165	170	175						
	A G E A E R L R A Y L E G T C V E W L R R Y L K N G N A T											
	–	–+	+	–	–	++	+	*	K7-65	K9-178	5F-1	28-13-3	K9-136	28-8-6
K^b									+[2]	+	+	+	+	+
bm1	—A—	YY							–	–	–	–	–	–
bm10				E—M—			E L		.[3]	.	–	–	.	–

* Carbohydrate moiety.
[1] Data are compilation of references 67, 76, 130, 56.
[2] Key: Reactivity in different studies: (+) greater than 25%; (±) 10–25%; (–) 0–10%.
[3] Not determined.

antibodies, K7-65 (67), 28-8-6 (132), K9-178 (67), 5F1 (134), and 28-13-3 (135), were mapped to amino acid changes at positions 152, 155, 156, 163, 165, 173, and 174 by loss of reactivity with cells from mutants bm1, bm4, or bm10 (Table 5). A single monoclonal antibody, K9-136 (67), appeared to map to a site created by interaction between the α1 and α2 domains; it showed weak or complete loss of binding to both bm3 (α1) and bm4 (α2).

The monoclonal antibodies that can be mapped to these regions (Tables 4 and 5) reveal different binding patterns on the various mutants, suggesting that they are detecting related but nonidentical epitope patterns. Regarding the antibody mapping data, chemically mutagenized variants selected for loss of reactivity by the above antibodies also show amino acid changes in these stretches. For example, preliminary analysis (P. Ajit Kumar, personal communication) of one such variant, R8.246 (see Table 2A) selected by loss of binding by EH-144, an α1 reactive monoclonal antibody has a single change at position 80 in its *K* molecule.

Summary of Structure-Function Studies on MHC Mutants

In summary, both in vivo and in vitro class-I variants have been studied as recognition elements for alloreactive and viral-restricted CTLs, and monoclonal antibodies. Not all the available mutants have been tested in all the virus restriction systems, nor has an extremely large number of monoclonal antibodies to K^b been examined; however, taken together, the studies demonstrate that:

1. Small changes in amino acid sequence in either the α1 or α2 domain can cause significant alterations in recognition by antibody or CTL.
2. Primary sequence similarity of different mutants is reflected in similar patterns of immune recognition by both the antibody and cell-mediated systems.
3. The majority of monoclonal antibodies to K^b recognize discrete, domain-specific sites present either in α1 or α2. These sites are localized to or influenced by polypeptide stretches in the α1 domain from amino acid residues 70 to 90, and in the α2 domain from 150 to 180.
4. CTLs recognize conformational determinants produced by apparent interaction of amino acid residues located in sites in either the α1 or the α2 domains of the class-I heavy-chain polypeptide.
5. The α3 domain does not appear to influence the conformational determinants (present in α1 and α2) required for CTL recognition.

Features of Class-I Molecules Relevant to MHC/*T Cell–Receptor Interactions*

The advances in protein sequencing and molecular genetic analyses have expanded our understanding of the overall structural properties of the

class-I molecules and the organization and interaction of their genes in the *MHC*. At the three-dimensional structure level, we have no information on the properties of these molecules that relates to interactions between the class-I protein and the T-cell receptor. Nonetheless, primary sequence data specifically with respect to the bm mutant changes, when correlated with antibody-binding and CTL-recognition data, suggest a number of features of the class-I molecule relevant to its recognition by the immune system.

RECOGNITION REGIONS Studies on the antigenic structures of proteins (136) conclude that antigenic determinants must be formed of, or assembled from, amino acid residues that are situated on the surface of the protein and are topographically adjacent. In addition, there are certain sequences that appear to be "immunodominant" for reasons intrinsic to the protein or the regulatory mechanism of the host.

Clearly, immunodominant or recognition regions must exist on class-I molecules to interact with the T-cell receptor. While there is no direct evidence, the data available on the K^b molecule on critical residues for antibody binding and CTL recognition permit speculation on possible recognition sites. The speculation is necessarily biased to the available data. Since the majority of monoclonal antibodies have detected clusters of amino acids in either the $\alpha 1$ domain (70–90) or $\alpha 2$ domain (150–180) we have defined these stretches as major recognition regions (Tables 4 and 5).

By "recognition region" we imply only that certain amino acid residues in such stretches may be in contact with an immune receptor or, possibly, may affect other residues that form a class-I molecule site for immune recognition. Since residues in the recognition regions interact with an immune receptor, it would follow that these stretches of residues are located on the surface of the protein domains. Consistent with their proposed surface exposure is the finding that both stretches contain a large number of charged hydrophilic residues, Arg, Lys, Glu, or Asp, and a carbohydrate moiety. In defining these regions we have arbitrarily chosen boundaries encompassing amino acid residues defined only by those K^{bm} mutant molecules that demonstrate altered binding by monoclonal antibodies. The mutant *K* molecules of the bm8 mice and "bg series" mice do not show altered antibody binding, and therefore the stretch of substitutions are not included as recognition regions, although the changes in the *K* sequence in these mutants are detected by CTL.

It is particularly relevant that several CTL-detected *HLA* variants have amino acid changes in the postulated recognition regions described for the K^{bm} mutants. For example, the peptide encompassing amino acids 147–157 has been altered in four *HLA* variants (53, 57, 82, 83, 137, 138). In addition, two *HLA* variants were found to have changes in the $\alpha 1$ recognition region at position 77 (and 152) (57) and another at positions 77, 80, 81 (52). It is

striking therefore that studies on variants from the human system, as discussed by Lopez de Castro et al (17), as well as the mouse system, identify similar immune dominant stretches of amino acids in class-I molecules. Of course, it is quite possible that as more data accumulate we may find evidence to establish the importance of other regions in *MHC* recognition.

Whereas individual antibodies appear to interact with either one or the other recognition region of the α1 and α2 domains, CTLs concordantly recognize sites determined by these two stretches. This could mean that the T-cell receptor interacts simultaneously with these separate immunodominant regions, since a change in either the α1 or α2 recognition region alters CTL interaction, or that stretches of polypeptide from each region of the class-I molecule interact to form a single combinatorial determinant. The interpretation that interaction of the α1- and α2-recognition regions forms a joint noncontiguous site is favored by the finding that one monoclonal antibody, K9-136, appears to recognize a site dependent upon juxtaposition of the two regions, since it has lost binding to both the K^{bm3} (alterations at residues 77 and 89) and the K^{bm4} products (alterations at residues 173 and 174).

An additional possibility could be that one of the postulated recognition regions is dominant, and the T-cell receptor interacts with this site—most likely the α2 region; this is suggested by the extraordinary biological effects of the changes found in bm1 (Table 3) and in many *HLA* variants (17). The site in the α1 region, while still accessible to monoclonal antibody as a site distinct from the α2 region, must have long-range allosteric effects on the conformation of the α2 recognition region. Consistent with this idea is the fact that the α1 domain lacks a disulfide bridge. Thus, the lack of constraint imposed by intradomain disulfide cross-linking might allow sufficient flexibility in the folding of the polypeptide strands of the α1 domain to permit interaction with polypeptide stretches of the α2 domain.

The sites defined by the bm mutants are clearly important for both monoclonal antibody and CTL recognition. The hypothesis that CTLs see sites on both the α1 and α2 domains or on an α1/α2 interaction site, and that antibodies see individual sites localized to a single domain, identifies a fundamental difference between recognition by the two systems. Further analysis using additional in vitro mutants of K^b will allow finer probing of the requirements for interaction between the T-cell receptor and a particular "site" on the *MHC* molecule. Precise topographical relationships must await the knowledge of the three-dimensional configuration of both the T-cell receptor and *MHC* class-I molecules.

SUMMARY

The class-I mutants have provided a model system for understanding the generation of diversity of the genes encoding the histocompatibility molecules *K*, *D*, and *L*, and the relationship of their structure to function. The complex nature of the alterations found in K^b molecules from mutant mice has been documented at the nucleic acid level for eight mutants. The clustered changes in the mutant genes are consistent with the hypothesis that genetic recombination between class-I genes generates the K^b mutants. Techniques using synthetic oligonucleotide probes to mutant DNA sequence demonstrated that other class-I genes were available as donors for interaction with the K^b gene to produce the mutations. Intriguingly, donor genes found in the *K* region (*K1*) and the *D* region (D^b), as well as the *Qa* regions (*Q4*, *Q10*), were capable of the interactions. The amount of genetic transfer to K^b from other class-I donor genes may range from a potential minimum of 5 nucleotides to a potential maximum of 95 nucleotides. Genealogical analysis of several bm mutants has further indicated that at least some, if not all, of the gene interaction events generating K^b mutations occurred during mitotic amplification of the germ cells. Genetic recombination among class-I genes occurring in nature to the extent observed for the K^{bm} mutants could readily generate mosaic transplantation genes containing sequences derived from other class-I genes. Thus, it seems likely that genetic interaction plays a major role in the diversification and ongoing evolution of the *MHC*.

The localization of altered amino acids in the in vivo mutant K^b molecules has directed our attention to recognition regions on the K^b product that play a major role in determining alloreactivity and *H-2* associative recognition. The replacement of one or a few amino acids in either of the postulated recognition regions located in the $\alpha 1$ domain (residues 70–90) or $\alpha 2$ domain (residues 150–180) can have marked effects on biological function. While the majority of monoclonal antibodies recognize epitopes in one or the other recognition region, CTL recognize determinants dependent on the apparent interaction of amino acids located in both regions. These overall conclusions are supported to a large extent by studies on mutants derived from several sources, i.e. spontaneous mutants, mutagen-induced somatic variants, and products of hybrid *H-2* genes.

Studies of in vitro variants can provide a more refined approach for analysis of structure-function relationships through the introduction of minimal biochemical changes. Loss variants will provide a unique approach to understanding the regulation of class-I gene expression and, in addition, information on those regions of the encoded products respon-

sible for directing these molecules to the cell surface. Clearly, analysis of variants produced by a variety of methods is essential to a complete understanding of the regions of class-I molecules that play a role in their expression and function.

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NOTE ADDED IN PROOF Additional sequence data on mutant K^{bm4} indicate that further changes at amino acid positions 162, 163, and 165 are present, which are not identical to the changes in K^{bm10}.

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CONTENTS

Ann. Rev. Immunol. 1986. 4 : 503–28

THE MEMBRANE ATTACK COMPLEX OF COMPLEMENT

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INTRODUCTION

Cell-killing mechanisms are essential components of host defense against infectious agents, parasites, and malignant cells. We distinguish humoral and cellular killing mechanisms. Complement utilizes specific proteins to assemble its killer molecule. Macrophages generate active oxygen radicals that injure target membranes. Lymphocytes, like complement, use protein molecules as tools in the cellular cytotoxicity reaction. As will be suggested at the conclusion of this chapter, considerable similarity between the structure of the cytolytic apparatus of complement and that of lymphocytes has recently come to light. An in-depth knowledge of the structure and function of the killer molecule of complement may therefore facilitate elucidation of the cell-killing mechanism of lymphocytes.

Complement encompasses 20 proteins, not including the various cell-surface complement receptors and regulatory proteins. Only 5 of these 20 proteins participate directly in cell killing—C5, C6, C7, C8, and C9—and none has enzymatic activity, proteolytic or lipolytic. Upon activation of C5, the 5 proteins interact in a sequential manner and fuse into a macromolecular organization, called the membrane attack complex or MAC. Fusion brings forth hydrophobic sites through which the complex inserts itself into the hydrocarbon core of lipid membranes. There it forms transmembrane channels, the largest of which constitutes tubular poly C9.

Activation of C5 is accomplished by a highly specific serine protease, C5 convertase, itself an assembly of three protein molecules. Thus, formation of the MAC is initiated enzymatically, but this enzyme does not participate in actual membrane attack, which is entirely a physicochemical process.

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The actual elucidation of the membrane attack mechanism began about 15 years ago when it was regarded for the first time as a problem of macromolecular protein chemistry. Electron microscopy has greatly facilitated progress in this field; in fact, the image of the MAC was visualized in the electron microscope long before the existence of the MAC became known.

HISTORICAL PERSPECTIVE

The first clear evidence that complement produced functional holes in cell membranes rather than membrane rupture was presented by Green, Goldberg and their associates in 1959 (1). Four years later, Humphrey & Dourmashkin (2, 3) reported their electron microscopic findings, that complement evoked structural membrane lesions. They described these lesions as membrane holes (top view) or hollow cylinders filled with negative stain (side view). These lesions were not affected by treatment with proteases but were removed from formaline-fixed membranes by lipid extraction with chloroform-methanol; this suggested that complement attack effected a structural change of the lipid bilayer rather than of membrane proteins.

In his one-hit theory of immune hemolysis, Mayer (4) had postulated in 1961 that a single membrane lesion was sufficient to cause lysis of an erythrocyte and that a single complement molecule at least at some stage of the reaction sequence suffices for production of this lesion. When it was found in 1964 that the number of ultrastructural lesions corresponded approximately to the theoretically predicted number of functional lesions, it was assumed that the visible membrane holes also constituted the functional holes (5).

In the 1960s the classical pathway of complement was shown to be composed of 11 proteins, but it was not until 1969 that knowledge of the functional organization of the pathway emerged (6). The conceptual distinction could now be made between the activation mechanism consisting of C1q, C1r, C1s, C2, C3, and C4 and the actual membrane attack mechanism comprising C5, C6, C7, C8, and C9. The experimental basis for the definition of C5-C9 as the membrane attack unit was furnished by Götze & Müller-Eberhard (7) and through an independent approach by Lachman & Thompson (8).

Since the five proteins were available in highly purified radiolabeled form, a molecular analysis of their mechanism of action was performed. In 1972 Kolb et al (9) reported that all five proteins become firmly bound to the target membrane as a compact complex containing equimolar amounts

of C5b, C6, C7, C8 and multiple molecules of C9. Binding of C9 was proposed to be a cooperative process (10).

Utilizing the available experimental information, Mayer (11) in 1972 proposed the doughnut model of the MAC according to which each of the five proteins contributes one molecule to form a large hydrophilic protein channel or doughnut within the lipid bilayer of a target membrane. This bold suggestion turned out to be essentially correct, except that the image of the annular structure is not evoked by C5b-9, but, as we know today, by poly C9. Direct evidence for the occurrence of a stable C5b-9 complex was furnished by Kolb & Müller-Eberhard (12), who in 1973 described the hydrophilic counterpart of the MAC, SC5b-9; subsequently, Bhakdi et al (13) in 1976 reported extraction of the MAC from the membranes of sheep erythrocytes lysed by complement. Mayer's doughnut model appeared to be verified when Tranum-Jensen et al (14) in 1978 found the extracted MAC to resemble morphologically the membrane lesion that Humphrey & Dourmashkin (3) had described as a hollow cylinder. As noted before, the assumption was made that all five proteins participated in the physical make-up of the cylinder. But just how five hydrophilic and physically different serum proteins create a cylindrical membrane structure was not addressed.

Between 1980 and 1982 it was found in this laboratory that the cylindrical structure of the MAC was due to C9 polymerization. It was shown that isolated C9 can undergo spontaneous polymerization to hollow tubular structures that resemble the MAC morphologically (15, 16). And, with SDS gel gradient electrophoresis, poly C9 was detected in 1982 for the first time as a constituent of the fully assembled MAC (17). It has now become clear that a cytolytically active C5b-9 site on the surface of a cell need not look like a membrane hole and thus that poly C9 is not an obligatory constituent of the MAC. The cytolytic potential of nontubular C9 oligomers within the MAC remains to be fully evaluated.

In 1980 Dourmashkin et al reported that human killer lymphocytes insert tubular structures into target membranes that morphologically resembled the MAC of complement (18). Subsequently, these tubular structures were also observed in cytolytic reactions involving mouse (19) and rat (20) lymphocytes.

THE MAC PRECURSOR PROTEINS

The five proteins that execute membrane attack are, in their native form, hydrophilic glycoproteins with molecular weights ranging from 70,000 to 190,000. Some of their properties are summarized in Table 1. C5 has a

molecular weight (M_r) of 191,000 (21) and consists of two disulfide linked chains, an α and a β chain, with respective M_r of 115,000 and 75,000 (22). The M_r of the carbohydrate of C5 is 5,200 (21). The primary structure of human C5 has been partially elucidated (23) and that of murine C5 (24) completely derived from cDNA sequence analysis. The amino acid sequence of C5 exhibits pronounced homology to that of C3, C4, and α2-macroglobulin. C5 lacks an internal thioester since the region corresponding to the thioester domain of C3 and C4, although homologous, shows a decisive difference in sequence. The structural gene of murine C5 has been assigned to chromosome 2 (25). C5 is the precursor of two biologically active fragments, C5a (M_r, 11,200) and C5b ($M_r \sim 180{,}000$), that arise when C5 convertase selectively cleaves the arginyl-leucine bond at position 74–75 of the α chain of C5. C5a is one of three complement-derived anaphylatoxins and a potent leukocyte chemotactic peptide (26). It also exhibits immunoregulatory activity in vitro, since it enhances the humoral T cell–mediated immune response (27). C5b in its nascent state (5b*) constitutes the nucleus in the assembly of the MAC. C5b* possesses a metastable binding site with specificity for C6. The metastability is probably due to the transient expression of hydrophobic sites, because C5b* forms aggregates in the absence of C6 that are not dissociable by high salt concentration but are completely dissociated in 1% SDS (21). Electron microscopy of negatively stained C5 revealed a multilobal, irregular molecular structure with estimated dimensions of 168 × 151 × 104 Å (21).

C6 and C7 have similar physical and chemical properties, suggesting that

Table 1 Properties of the proteins of the membrane attack pathway

Protein	M_r	Subunits	M_r carbohydrate	s-Rate	Serum concentration (μg/ml)
C5	191,000	α: 115,000 β: 75,000	5200	8.7S	70 (55–80)
C5b	180,000	α': 104,000 β: 75,000	3000	7.5S	
C6	120,000	Single chain	4000	6.0S	64 (54–72)
C7	110,000	Single chain	6000	5.6S	56 (49–70)
C8	151,000	α: 64,000 β: 64,000 γ: 22,000	na	8.0S	55 (43–63)
C9	71,000	Single chain	5500	4.7S	59 (47–69)
S-protein	83,000	Single chain	na	4.0S	505 (418–600)

na: Information not available.

they evolved from a common ancestral gene. In fact, a close linkage between the loci for both proteins has become apparent from family studies of the genetic polymorphisms of C6 and C7 (28). Further evidence for linkage was provided by the observation of an inherited combined C6, C7 deficiency (29). Both are single chain glycoproteins (30). The reported M_r values for C6 range from 104,800 to 128,000 and for C7 from 92,400 to 121,000 (31). C6 is synthesized primarily in the liver (32), whereas the site of C7 synthesis is uncertain. The human histiocytic cell line U937 has recently been shown to synthesize C7 (33). C6 had been claimed to be a serine protease because its hemolytic activity appeared to be inactivated by serine protease inhibitors (34). Inactivation of C6 by DIFP (diisopropyl fluorophosphate) or PMSF (phenylmethanesulfonyl fluoride) could not be confirmed (35). The notion that C6 might be a protease also came from the observation that the α chain of C5 is cleaved within the acid-induced, hemolytically active complex of C5 and C6 (36). It has become abundantly clear that C6 is not a serine active site enzyme. The serine protease activity associated with preparations of isolated C6 could be separated from C6 hemolytic activity and characterized as thrombin (37). This study also established that the hemolytically active, acid-induced C5,6 complex contains an intact C5 α chain if the complex is prepared from protease-free C5 and C6. Neither C6 nor C7 has been structurally elucidated, and although attempts have been made in several laboratories, cDNA for the two proteins has not been successfully cloned.

C8 is an unusual protein with respect to its structure and resistance to irreversible denaturation by protein denaturants. It consists of three nonidentical chains: The α and γ chains are disulfide linked, whereas the β chain is noncovalently associated with the former two (38, 39). This exceptional subunit structure is, in part, explained by the recent finding of separate loci encoding the C8α-γ and C8β subunits (40). C8 possesses an M_r of 151,000; the M_rs of the α, β, and γ chains are respectively 64,000, 64,000, and 22,000 (41). Denaturation of C8 by 6 M guanidine hydrochloride and 8 M urea or 0.2% SDS was almost completely reversible by removal of the denaturants (41).

C9 consists of a single polypeptide chain, has an M_r of 71,000, contains 7.8% carbohydrate, and has a blocked NH_2-terminus (42). A significant advance was made toward understanding the structure of C9 in relation to its function when it was shown that the molecule can be cleaved by α-thrombin at a single site into a 34,000 M_r hydrophilic fragment, C9a, and a 37,000 M_r hydrophobic fragment, C9b (43). C9a represents the NH_2-terminal and C9b, the COOH-terminal segment of the molecule. The fragments remain noncovalently associated and can be separated in the presence of SDS. The hydrophilic C9a may be important for the solubility

of native C9 and for its interaction with C5b-8; the hydrophobic C9b may serve to anchor the MAC or poly C9 within the target membrane. Recently, the entire amino acid sequence of human C9 has been derived from the sequence of C9 cDNA isolated from a liver cDNA library (44). The deduced C9 protein consists of 537 amino acid residues, and the α-thrombin cleavage site was identified as a histidyl-glycine bond between residues 244 and 245. A hydropathy analysis of the amino acid sequence confirmed that the primary structure of C9 has an amphipathic organization. However, C9 lacks the transmembrane domain of 20–30 hydrophobic and nonpolar amino acids that is characteristic of membrane-spanning polypeptides. As a protein capable of forming a hydrophilic transmembrane channel, the hydrophobic phospholipid binding region is, not unexpectedly, a function of the tertiary and quaternary conformation of the molecule. Both C9a and C9b contain carbohydrate (43), but only one typical carbohydrate attachment site, Asn-X-(Ser/Thr), was identified (44). The sequence Asn-Glu-Thr occurs in the C9b portion of the molecule, which suggests that the asparaginyl residue in position 256 may be the carbohydrate attachment site in that domain. The carbohydrate moiety of C9a may be attached to a hydroxyamino acid. C9a contains 15 and C9b, 8 cysteinyl residues (44). Since no free sulfhydryl groups could be detected in C9 (45), it is likely that 1 sulfhydryl group in C9a is substituted and that all other groups in the C9 molecule are engaged in disulfide bonds. C9 cDNA was also cloned and sequenced by Stanley et al (46), and these authors noticed that C9 contains a 39-residue segment (residues 91–122) that is homologous to the major repeat unit in the NH_2-terminal position of the low density lipoprotein receptor. Lately, another homology was pointed out between a sequence near the COOH-terminus of C9 and an NH_2-terminal stretch of residues in urokinase and tissue plasminogen activator (47).

S-protein is the primary MAC-inhibitor of serum. It binds to the metastable membrane binding site of the nascent MAC and prevents C9 polymerization (48). The inhibitor ($K_i = 0.49\ \mu m$) was isolated from human serum and characterized as an 80,000-M_r single-chain glycoprotein with electrophoretic characteristics of an α globulin (49).

THE C5 CONVERTASES

There are two C5 convertases, one for the classical and the other for the alternative pathway. They are metallo-serine proteases consisting of a nonenzymatic subunit, either C3b or C4b, and the catalytic site–bearing subunit, Bb or C2a. They are basically C3 convertases and to act on C5 require a helper molecule, C3b (50–52). Its task is to change the conformation of C5 in such a way that the peptide bond 74–75 of the C5 α

chain becomes accessible to the catalytic site and C5 can be cleaved to C5a and C5b. Although the two enzymes are composed of different proteins, they exhibit considerable structural homology. Cloning and sequencing of DNA coding for C3 (53–55), C4 (56), Factor B (57), and C2 (58) revealed sequence homologies between C3 and C4 and between Factor B and C2. Factor B, C2, and C4 are products of the class-III MHC genes (59), whereas the C3 gene is not located within the MHC but resides on chromosome 19 in man (60). The classical C5 convertase, C4b,2a,3b (50), and the C5 convertase of the alternative pathway, C3b,Bb,C3b (51, 61), are labile enzymes with a half-life at 37°C of 2–3 min. The classical enzyme could be stabilized utilizing iodine treated C2 (62) and the alternative pathway C5 convertase by using Ni^{++} (63) instead of Mg^{++} for enzyme formation. The stable form of the enzymes was amenable to physical analysis including electron microscopy. In both enzyme complexes the catalytic subunit was visualized as a dumbbell-like two-domain structure, each sphere measuring 42–45 Å in diameter (64). Since only one domain (binding domain) can be seen in contact with the nonenzymatic subunit, the other, freely projecting domain may bear the catalytic site, which in the case of Bb has been located to the C-terminal half of the molecule (57). Due to the internal thioester in C3 and C4, nascent C3b and C4b can establish covalent bonds with hydroxyl or amino groups (65), enabling the C5 convertases to anchor themselves firmly to the surface of target cells.

THE REACTION SEQUENCE

The relevant product of C5 convertase action is nascent C5b (C5b*). It is labile but capable of forming a stable, hydrophilic, bimolecular complex with C6. C5b,6 remains loosely bound to C3b until it binds to C7 and induces it to undergo a hydrophilic-amphiphilic transition. Hydrophobic binding regions for phospholipid are thereby exposed, and the trimolecular complex is endowed with a metastable membrane binding site. Membrane-bound C5b-7 inflicts no harm on a cell but marks it for further assault. C5b-7 constitutes the receptor for C8 that binds and processes C8 so that its α chain can enter the target membrane. C8 then binds C9 and nonenzymatically catalyzes its polymerization, during which C9 undergoes hydrophilic-amphiphilic transition and considerable molecular reorganization. The final product is tubular poly C9 with C5b-8, the polymerizing unit, firmly attached to it. Alternatively, C5b-9 may contain nontubular C9 oligomers. As the assembly progresses, the number of phospholipid binding sites increases. Whereas C5b-9 creates the large complement channel, C5b-8 produces a small membrane pore sufficient to lyse erythrocytes but by and large unable to kill a nucleated cell. The MAC-inhibitor or S-protein, which

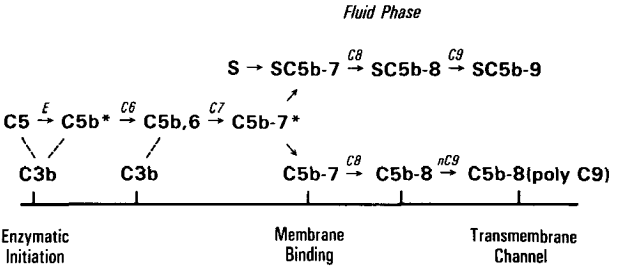

Figure 1 Schematic representation of assembly of the MAC and its control by S-protein. The asterisks denote the metastable forms of C5b and C5b-7, respectively.

is a serum glycoprotein, is capable of binding to the membrane binding site of metastable C5b-7, thereby forming the soluble SC5b-7 complex. The complex will accept C8 and C9 to form hydrophilic SC5b-9, but will not allow C9 polymerization. Figure 1 shows a schematic representation of the reaction sequence.

THE METASTABLE MEMBRANE BINDING SITE

The first direct membrane contact by the forming MAC is made through metastable C5b-7. And the subunit of the trimolecular complex that furnishes the binding site is, in all probability, C7. When metastable C5b, the half-life of which is 2.3 min at 37°C (50), binds C6, the stable C5b,6 complex is created which has an s-rate of 11.5S and an M_r of 328,000 (66). Upon binding of C5b,6 to C7, the membrane binding site is generated, and the C5b-7 complex anchors itself firmly in the lipid bilayer of the target membrane by hydrophobic interaction. The lifetime of the transient membrane binding site of C5b-7* has been estimated to be less than 10 ms (67). Bound to single bilayer phospholipid vesicles, C5b-7 is visualized by electron microscopy as a leaflet extending approximately 210 Å above the vesicle surface which is attached to the membrane through a 40 Å long, thin stalk (68). Often V-shaped bileaflet images are seen with a single stalk that appear to be C5b-7 dimers. If binding to a membrane does not occur, C5b-7* forms soluble protein micelles that have an s-rate of approximately 36S (68). The soluble aggregates are imaged in the electron microscope as flower-like structures in which the C5b-7 monomers remain clearly distinguishable, three to six leaflets terminating in a pedicle at the center of the flower, which indicates that this is the region of hydrophobic interaction. Two independent experimental approaches strongly suggest that the hydrophobic binding site of C5b-7 is located in C7.

First, colloidal gold particles were coated with either C5b,6 or C7 and

then exposed to the other protein not present on their surface. Gold-C7 conjugates treated with C5b,6 appeared in the electron microscope as individual particles with finger-like protein structures protruding from their surface and resembling C5b-7 protein micelles. In contrast, when the gold-C5b,6 conjugates were treated with soluble C7, clusters of gold particles were visualized in which the individual particles were linked up by proteinacious material (68). This experiment was interpreted to show that a C7 molecule adsorbed to a surface simply binds a molecule of C5b,6, whereas free C7, on binding to C5b,6 adsorbed to particles, is activated and then enters into hydrophobic C7-C7 interaction.

Second, exposure of isolated C7 to 4 mM DOC at 37°C for 10 min resulted in complete loss of hemolytic activity and dimerization of the protein (69). The effect of DOC treatment was strongly temperature-dependent and increased the s-rate from 5.5S to 7S, the M_r from 120,000 to 230,000, and β-pleated sheet structure by 18%. The dimer possessed hydrophobic surface domains that are evidenced by its ability to bind approximately 82 mol of DOC per mol of dimer. Removal of the detergent rendered the C7 dimer insoluble, but readdition of detergent solubilized the precipitated protein, suggesting that the C7 dimer behaved as would a membrane protein. In fact, the dimer readily incorporated itself into lipid vesicle membranes and rendered them leaky (69). Because dimer formation was induced by detergent, it may be assumed that the dimerization site in the C7 molecule has polar properties. None of the changes in properties observed with C7 occurred with C5 or C6 upon treatment with DOC. Thus, C7 appears to be constructed so as readily to undergo hydrophilic-amphiphilic transition.

The available information suggests that in the physiological pathway C5b,6 acts on C7 as does DOC; it converts C7 to an activated, amphiphilic state. C7 emerges as a highly versatile molecule with several different functional sites: Native C7 has a binding site for C5b,6. When bound by C5b,6, activated C7 has multiple phospholipid binding sites. In the aggregate these constitute the hydrophobic membrane binding site of C5b-7*. It also expresses a protein binding site, probably polar, by means of which C5b-7 can dimerize.

TETRAMOLECULAR C5b-8

Native C8 has no detectable affinity for membranes and is therefore entirely dependent on the mediating function of C5b-7, which determines the site of membrane attack and serves as a receptor for the C8. Membrane-bound C5b-7 not only binds C8, it also affords its insertion into the membrane. The recognition site on C8 for C5b-7 has been located to the β chain of C8 (70).

This chain was shown to bind to C5b-7 in absence of the α-γ subunit and with the same affinity as native C8. The α-γ subunit did not attach to C5b-7 without the mediation of the β chain (70, 71). This initial contact probably brings a second site of the receptor into play that enables C8 to undergo the necessary conformational rearrangement to allow the α chain to penetrate into the hydrophobic core of the lipid bilayer. The function of the second or C8 processing site would be greatly facilitated if it were located near the membrane surface and on a structure that itself is partially inserted into the membrane. It is quite possible, therefore, that this site is located on C7. Using membrane-restricted, photoactivatable probes in lipid vesicle or erythrocyte membranes, it was shown that the α-γ subunit (72) and, more specifically, the α chain of C8 (73) were primarily interacting with the hydrocarbon phase. Electron microscopy of C5b-8 bound to erythrocytes made visual the foliaceous, branched structures that radiated from a central pedicle (74). However, on small phospholipid vesicles, monomeric C5b-8 could be imaged as a 250-Å-long and 50–140-Å-wide rodlike structure with a rather polymorphic appearance (75). The C5b,6 subunit was detected at the end of the rod, distal to the lipid binding site, using avidin-coated gold particles recognizing biotinyl C5b,6. C5b-8 serves to concentrate C9 on its surface and thereby to facilitate its oligomerization or polymerization. Binding of C9 occurs via the C8 portion of C5b-8. By what mechanism the complex catalyzes the self-association of C9 is unknown. Soluble C8 binds only one molecule of C9 even when C9 is in considerable molar excess (17).

COMPOSITION AND STRUCTURE OF C5b-9

The MAC is heterogeneous with respect to size and composition. The basic composition of the MAC is described by the formula $C5b_1, C6_1, C7_1, C8_1, C9_n$, where n can vary between 1 and 18. Hence, the minimum M_r of the MAC ranges between 660,000 and 1,850,000. That multiple copies of C9 can be incorporated into C5b-9 first became apparent when the binding of differentially radiolabeled C8 and C9 to target cells was quantitated (9). The binding curve and hemolytic dose response curve were sigmoidal, indicating cooperativity between multiple C9 molecules during this reaction step (10). The C9-binding capacity of C5b-8 was found to average 15.4 molecules per C8 molecule with a range of 12 to 21 (17). SDS-resistant macromolecular C9 was first detected in the MAC by employing SDS polyacrylamide gel gradient (2.5–10%) electrophoresis (17, 76). This form of C9, which has a molecular weight of 1.1 million, was shown to resemble spontaneously formed poly C9 (see below) by electron microscopic, physical, and immunochemical criteria (17, 15). There is no doubt that the image of the cylinder-like membrane lesion caused by complement is

evoked by poly C9. Poly C9 of the membrane-bound MAC extends 120 Å above the surface of the membrane; it has an inner diameter of 110 Å; and it terminates at its upper, hydrophilic end in a 30 Å-thick annulus, which has an outer diameter of 210 Å (14, 17, 74). The C5b-8 subunit appears firmly attached to the poly C9 tubule and extends 160–180 Å above its annulus as a 50–140 Å-wide elongated structure (75). The total length of the structure is thus 280–300 Å above the surface of the membrane.

The structure of the MAC is determined by the molar C9/C5b-8 ratio. When the MAC was assembled on vesicles or erythrocytes and the molar C9/C5b-8 ratio was 1 to 3, no poly C9 was detectable by SDS polyacrylamide gel electrophoresis (77), and no complement membrane lesions were seen on electron microscopy (17). Instead, large protein aggregates were observed that probably represented a C5b-9 network maintained by C9-C9 interactions. When this molar ratio was 1 to 6 or 12, the proportion of C9 present as poly C9 was 35% and 72%, respectively (77). At these ratios discrete ring structures were observed on the cell surface by electron microscopy; the structures appeared unaggregated and well separated from each other (17). Incompletely polymerized or oligomeric C9 is not readily visualized by the negative staining technique and not detected by gel electrophoresis in presence of SDS because it is not SDS-resistant. However, oligomeric C9 is stable in DOC and can therefore be estimated in the DOC-solubilized MAC (78). The C9 content relative to C5b-8 is inversely proportional to the size of the aggregates. This may indicate that oligomeric C9, in contrast to poly C9, promotes aggregation of C5b-9. In fact, poly C9 was detected primarily in the unaggregated material of relatively low M_r (77). Thus, C5b-9 will either aggregate to larger clusters when the C9 multiplicity is low or form poly C9 containing MAC monomers when the C9 multiplicity is high. Obviously, SDS-resistant poly C9 cannot be regarded an obligatory constituent of the MAC, and C5b-9 does not necessarily manifest itself as the typical ultrastructural membrane lesion.

C9 polymerization within the MAC is distinct from spontaneous poly C9 formation in that it proceeds in the absence of metal ions. Under these conditions, the rate of poly C9 formation by C5b-8 is 10,000-fold greater than that of spontaneous poly C9 formation (77).

SPONTANEOUS C9 POLYMERIZATION

In isolated form, C9 has the propensity to aggregate and to undergo hydrophilic-amphiphilic transition that results in cyclic polymerization. Spontaneous poly C9 formation is presumably an unphysiological reaction, but its analysis may aid our understanding of the physiological

process of MAC assembly. Poly C9 is a tubular structure which has a hydrophilic annulus of 30 Å thickness at one end and is lipophilic at the other end. The height of the tubule is 160 Å, the inner diameter 100 Å, and the wall thickness approximately 20 Å. Of the C9 12–18 molecules are incorporated into tubular poly C9, also defined as an approximately 1.1 million M_r complex that is resistant to dissociation by SDS and reducing agents. Poly C9 possesses more β-pleated sheet structure than native, monomeric C9, and it expresses antigenic determinants that are not detectable on its precursor (15–17). In the ultracentrifuge poly C9 exhibits polydisperse sedimentation behavior with s-rates ranging from 27S to 250S. By electron microscopy, the aggregates were visualized as arrays in which the annuli of adjacent tubules pointed in opposite directions, and in which the smaller ends of the tubules combined side-by-side and overlapped by about 40 Å. These aggregates could be dissociated to 27S unaggregated poly C9 by 1% DOC or 0.1 M octylglucoside (78).

Through the lipophilic end the assembling tubule is capable of inserting itself into the membrane of phospholipid vesicles, to render them leaky (15). The hydrophobic portion of the poly C9 tubule corresponds to the C-terminal region (C9b) of its subunits (79). To measure the size of the poly C9 channel, the marker retention assay was used in which high molecular weight proteins of defined molecular diameter are entrapped in lipid vesicles. Preformed poly C9 was incorporated into the membrane of the vesicles during their preparation. Alcohol dehydrogenase, which has a Stokes' radius of 45 Å, escaped through the poly C9 channel, whereas C3, which has a Stokes' radius of 51 Å, did not (80). The functional diameter of the poly C9 channel is therefore between 90 Å and 102 Å, which is in excellent agreement with the electron microscopic image.

Poly C9 was detected serendipitously by electron microscopy. The environmental conditions employed initially were unfavorable, and several days of incubation at 37°C were needed to yield a small amount of poly C9 (16, 15). However, a systematic exploration showed that spontaneous polymerization of isolated C9 commenced within minutes and reached its final extent within a few hours if the salt concentration was low (20 mM), the pH between 7.5 and 9.0, and if metal ions were present; the 1–2 mM Ca^{++} or Mg^{++} or 50 μM Zn^{++} were most effective. EDTA completely prevented polymerization (81–83).

The molecular weight of poly C9 was determined by sedimentation equilibrium ultracentrifugation in presence of 0.2% SDS or 1% DOC and found to be $1{,}060{,}000 \pm 118{,}000$, exclusive of the bound detergent. It was also determined by electron scattering of unstained poly C9 in the scanning transmission electron microscope, and this method yielded a value of $1{,}078{,}000 \pm 194{,}000$. Since negative-staining electron microscopy revealed a

variation of the inner diameter of poly C9 between 90 and 120 Å, poly C9 is assumed to be heterogeneous with respect to the number of protomers (84).

A comparison of the physical properties of poly C9 and monomeric C9 (Table 2) reveals that they differ most strikingly in molecular dimensions. Electron microscopy showed that the long axis of monomeric C9 is approximately 80 Å and the height of the poly C9 cylinder is 160 Å. It was proposed therefore that the C9 molecule is constructed to undergo constrained unfolding and to assume an elongated rodlike structure under nondenaturing conditions (15). To test this possibility, native C9 was treated with an excess of succinic anhydride at room temperature in order to substitute lysine residues with negatively charged, mutually repellent succinyl groups (83). Native C9 and succinyl-C9 were then compared by analytical ultracentrifugation and molecular sieve chromatography on Sephacryl S-300. Succinylation reduced the s-rate of C9 from 4.7S to 2.6S and increased the apparent molecular weight from 80,000 to 440,000. The pronounced increase in Stokes radius from 38 Å to 61 Å indicates a considerable increment in molecular asymmetry upon succinylation of C9. This observation is consistent with the notion that C9 is capable of changing its conformation from globular to rodlike. Electron microscopic analysis of monomeric C9 has recently revealed one type of image that has the appearance of a small "c" with one half of the structure considerably thicker than the other (F. J. Chiu, H. J. Müller-Eberhard, unpublished). This image would suggest that the molecule is poised to open itself up.

The question arises as to the molecular events that are a prerequisite for polymerization. Poly C9 formation is strongly temperature dependent. The optimal temperature lies between 30°C and 40°C, virtually no polymerization occurring below 15°C and above 50°C (82). The activation energy for heat-induced polymerization of C9 in solution was reported to be 40 kcal mol^{-1} (16) compared to 13 kcal mol^{-1} for polymerization by C5b-8-

Table 2 Physical properties of C9 and poly C9

	C9	Poly C9
M_r	71,000	1,100,000
s-Rate	4.7S	27S
Subunits	1	12–18
Dimensions:		
Length	~80 Å	160 Å
Width	~55 Å	~210 Å
α helix	24%	22%
β sheet	32%	38%

bearing erythrocyte ghosts (85). Therefore, native C9 was examined by analytical ultracentrifugation at a temperature (15°C) not sufficient for polymerization. The following observations were made (83): In the presence of EDTA, C9 sedimented as monomer (4.3S); in the presence of 50 μM Zn^{++} or 1 mM Mg^{++}, C9 behaved as dimer (5.9S), which dissociated to the monomer in 150 mM NaCl. When, in the presence of metal ions, the protein concentration was raised from 250 to 1,000 μg/ml, the dimer and tetramer (9.5S) were seen. Thus, self-association of C9 is metal-ion and protein-concentration dependent, ionic and reversible. Since conditions promoting reversible C9-C9 interaction at 15°C allowed poly C9 formation at 37°C, reversible oligomerization appears to be a prerequisite for tubular poly C9 formation. It may be hypothesized that in the spontaneous polymerization of C9 the metal ions bring about a proper intermolecular orientation so that specific recognition between certain residues can occur. At an elevated temperature this leads to structural rearrangements of two or more C9 molecules and a tight intermolecular fit or fusion.

In the physiological reaction C9 polymerizes in the absence of metal ions. It is proposed therefore that C5b-8 induces the critical orientation between two or more C9 molecules, which then leads to unfolding of C9, insertion into the lipid bilayer, and polymerization. C5b-8 reduces the activation energy of C9 polymerization and greatly enhances its rate, thus acting as a catalyst. Also, whereas polymerizing C9 can attack lipid vesicles in absence of C5b-8, it cannot lyse biological membranes unless C5b-8 is present on their surface.

The unusual stability of poly C9 was demonstrated by its resistance to boiling in 2% SDS and reducing agents (78). However, its dissociation into 70,000 M_r monomers by incubation at 60°C in 8 M guanidine thiocyanate for 3 hr suggested that the intersubunit forces holding C9 together are noncovalent. More recent studies suggest that the property of SDS-resistance of poly C9 may be induced by boiling the sample prior to SDS polyacrylamide gel electrophoresis. When poly C9 was first succinylated and then subjected to SDS polyacrylamide gel electrophoresis, it completely dissociated to monomeric succinyl-C9 (83).

CONTROL OF MAC FORMATION

The enzymes that initiate MAC assembly are controlled by three plasma proteins and, at least on the surface of erythrocytes, by two membrane proteins. The C3b of both C5 convertases that is instrumental in the modulation of C5 and the binding of C5b,6 is inactivated by Factor I with the help of either Factor H or CR1 (86). The subunits of the C5 convertases

that contain the enzymatic site are efficiently dissociated and thereby inactivated by DAF (86, 87).

The S-protein of plasma (88) competes with membrane lipids for the metastable binding site of C5b-7, and by binding to the complex, it prevents its attachment to the cell surface. The inhibition constant, K_i, is 0.48 μM, which is less than one tenth of the plasma concentration of the protein (49, 66). The resultant SC5b-7 complex contains three molecules of S-protein (M_r = 80,000) and has an s-rate of 18.5S and a molecular weight of 668,000. The hydrophilic complex binds C8 and three molecules of C9 to form SC5b-8 (s-rate, 20.5S; M_r 820,000) and SC5b-9 (s-rate, 23S; M_r 1,030,000) (88, 66). In addition to blocking the membrane binding site, S-protein also prevents polymerization of C9. Purified SC5b-9 lacks poly C9 as revealed by electron microscopy and SDS polyacrylamide gel electrophoresis. Poly C9 formation that occurs on mixing of purified C5b,6, C7, C8 and excess C9 was inhibited by the addition of purified S-protein in a dose-dependent manner (48). The C9 within SC5b-9 was in the native state, as evidenced by its ability to transfer to EAC1-8 and to lyse these cells (89). By removal of the S-protein the hydrophilic SC5b-9 complex can be transformed into amphiphilic C5b-9 that resembles the MAC with respect to ultrastructure, neoantigenic determinants, lipid binding regions and the ability to incorporate into lipid bilayers (90, 91).

At least on the surface of erythrocytes, yet another regulatory principle appears to operate. C5b-9 is much less efficient in lysing homologous erythrocytes than it is in lysing heterologous cells. Yamamoto (92) attributed the phenomenon of homologous species restriction specifically to C9. Others proposed that it is due to a C8 binding protein on human erythrocyte membranes (93). Lately Houle & Hoffmann (94) reported evidence that homologous restriction involves a serum factor that is adsorbed by homologous erythrocytes on exposure to serum. J. J. Houle, E. M. Hoffmann, and A. F. Esser (personal communication, 1985) now believe that this protecting serum factor is, in fact, C9. Hänsch et al (95) studied the lytic effect of C8 and C9 of five different species on the erythrocytes of the same five species and found that in all cases lysis was least efficient when C8 and C9 were homologous with respect to the target cell species. Inefficient lysis in the homologous systems was not due to failure of C9 to bind to the target membrane but was attributed to impaired channel formation by C9 (96).

A 38,000 M_r protein has been isolated from whole human erythrocyte membranes solubilized in 2% DOC by adsorption to and subsequent elution from a Sepharose-C9 column. The protein was incorporated into liposomes and was found to inhibit the liposome swelling assay when

C5b,6, C7, C8, and C9 were added. It also inhibited the poly C9 channel (97). The results suggest that there is a protein in human erythrocyte membranes that can block the channel-forming ability of the MAC.

THE MAC CHANNELS

Until recently there was considerable dispute as to whether the MAC contained within itself a hydrophilic protein channel that accounted for the cytolytic action of the MAC (doughnut model) (11), or whether possibly it caused the formation of hydrophilic lipid channels, i.e. a weakening of membrane structure with breakdown of the permeability barrier (leaky patch model) (98). It has now become clear that the MAC functions through both mechanisms.

The MAC possesses high affinity binding sites for 1,400 mol of phospholipid per mol of complex (99). On insertion of the MAC into the bilayer of phospholipid vesicles (72, 79) or of erythrocyte membranes (73), all constituents of the complex came in contact with lipid; this is evidenced by positive labeling with photosensitive membrane-restricted probes. However, the C9b portion of C9 and the α chain of C8 were identified as the primary contributors of the intramembrane peptide domains of the MAC. The penetration of the MAC into flat lipid bilayers and its impact on the order of the bilayer were determined by electron paramagnetic resonance spectroscopy using spin-labeled membrane probes (100). The observed spectral changes were interpreted to indicate that the strong hydrophobic interaction of C5b-9 with membrane phospholipids overcomes the cooperative interactions between the ordered lipid molecules. As a result, the bilayer lipids undergo a reorientation into micellar domains surrounding the C5b-9 complex. Lipid molecules adjacent to those bound to C5b-9 must also undergo reorientation and assume a corresponding configuration. A lipid bilayer so modified no longer constitutes a permeability barrier because packing defects at the boundary between different lipid phases allow transmembrane ion flow to occur. Thus, reorientation of bilayer lipids in a membrane creates a leaky patch. The formation of multiple, confluent leaky patches may facilitate membrane disassembly by C5b-9. The instability of the lipids in a C5b-9 modified membrane may result in ejection from the membrane of C5b-9 phospholipid complexes in the form of lipoproteins that are soluble in an aqueous milieu. This may explain the membrane effects of C5b-9 that are independent of channel formation, such as the release of phospholipid from *Escherichia coli* (101) and liposomes (102), and the dissolution of phospholipid vesicles and of viral envelope membranes (103). The MAC shows no lipase activity that could effect

covalent degradation of phospholipids and thus be responsible for weakening of membrane structure (135).

A functional membrane pore is already formed at the C5b-8 step of MAC assembly. Formation of the pore is slow, requiring several hours at 37°C, and the channel diameter is below 30 Å (80, 104, 105). C5b-8 lyses erythrocytes and causes transient release of ions from nucleated cells, but it does not kill nucleated cells (106), with the rare exception of undifferentiated human U937 histiocytes (107). The formation of C5b-8 channels suggests that secondary rearrangements of the C5b-8 complex have to occur, or possibly aggregation of two or more complexes within the target membrane may be required.

The size of the functional transmembrane pore produced by C5b-9 is highly variable, even on the same membrane, and dependent on the C5b-9 density and the molar C9/C5b-8 ratio. Recall that the theoretical M_r of the unaggregated MAC varied between 660,000 and 1,850,000 depending on the number of C9 molecules incorporated. If this number is low, the functional pore size may be small (80, 108); if it is high, formation of poly C9 tubules will be favored, and the pore diameter may be as large as 100–110 Å (80, 98, 109–112). The liposome swelling assay was used to reveal recently that upon addition of C9 to C5b-8-liposome complexes (from 1–12 C9 molecules per C8 molecule), the apparent channel size increased in proportion to the number of C9 molecules added (80). When there were 12 C9 molecules per C5b-8, the channel size was indistinguishable from that of poly C9.

That the channel structures of the MAC are capable of penetrating the target membrane to establish a transmembrane communication with the intracellular or intravesicular space was shown by several independent techniques. An open channel between the hollow core of the MAC and the vesicle interior was visualized by three-dimensional analysis of MAC-phospholipid vesicle complexes using electron microscopy (113). C9 could be cross-linked by entrapped transglutaminase from the interior of resealed erythrocyte ghosts bearing the MAC (114). A monoclonal antibody to C9 that did not bind to the MAC when added extracellularly inhibited complement-dependent marker release when sealed inside erythrocyte ghosts (115).

Lysis of nucleated cells by the MAC is a multiple hit phenomenon (116) partly due to the cell's ability to eliminate the inserted channels (117, 118). Whether the poly C9 channel or the C5b-9 network is more effective in bringing about lethal membrane damage despite cellular defense and membrane repair is not known. Parenthetically, it should be mentioned that killing of a rough strain of *E. coli* by complement required a high C9 to

C5b-8 ratio and poly C9 formation (119). Channel elimination and thereby recovery of nucleated cells from MAC attack has been shown to be due to removal of the MAC from the cell surface by vesiculation (121, 120). It was also found that the MAC causes a rapid increase in intracellular free calcium ion concentration long before cell lysis becomes detectable and that thereby certain functions of nucleated cells may be activated (122). For instance, the MAC on polymorphonuclear leukocytes was shown to stimulate the production of active oxygen metabolites (121); and on Ehrlich ascites tumor cells (123) and peritoneal macrophages (124), it activated the formation and release of arachidonic acid derivatives. The triggering of the arachidonic acid pathway in human leukocytes with generation of leukotriene B4 has also been shown to be effected by C5b-8 and attributed to calcium influx through the small 5b-8 membrane channel (125).

COMPARISON OF THE MAC WITH THE CYTOLYTIC MECHANISM OF LYMPHOCYTES

The killing mechanism of lymphocytes has been enigmatic for a long period of time and still remains to be fully elucidated. However, in 1980 Dourmashkin, Henkart, and their associates found that killer lymphocytes can elaborate a tubular structure that inserts into target membranes and that bore striking morphological resemblance to the MAC of complement (18). This tubular structure is currently regarded as the principal candidate for the cytolytic mechanism of CTL, NK, and K cells and has been observed in cytolytic reactions involving human (18), rat (20), and mouse lymphocytes (19). By electron microscopy the killer lymphocyte–derived membrane attack complex (LMAC) has the same height as poly C9 (160 Å), but a larger inner diameter, 160 Å instead of 100 Å. A smaller version of the complex has also been described. It is shorter than 160 Å and has an inner diameter of 60–70 Å (19). The precursor protein of the cylindrical structure, which may be referred to as membrane attack protein (MAP) (126), is contained in the cytoplasmic granules of killer lymphocytes (20, 127, 128) that have been isolated and found, in the presence of Ca^{++}, to lyse sheep erythrocytes or certain nucleated cells (20, 128) and to cause marker release from lipid vesicles (129). The MAP is also referred to as cytolysin, lymphopore, or perforin.

Earlier studies have shown the functional membrane pore produced by killer lymphocytes to be larger than that produced by complement (136). Using partially purified rat MAP, researchers showed the release of carboxyfluorescein from lipid vesicles to be highly efficient, and the initial

rate and final extent of release dose-dependent (129). Ca^{++} could be replaced by Sr^{++}, but not by Ba^{++} or Mg^{++}.

After disruption of isolated cytoplasmic granules of the mouse CTL cell line B6.1 in 2 M NaCl, and extensive ultracentrifugation to remove particulate matter, a soluble protein of $M_r \sim 66{,}000$ was isolated by Masson & Tschopp (130) that they identified as mouse MAP. In presence of Ca^{++} it exhibited hemolytic activity and formed tubular LMAC on lipid vesicles.

A C9-related protein was recently detected in isolated cytoplasmic granules of human large granular lymphocytes (LGL) which were maintained in culture for 2–6 weeks with regular addition of IL-2 (131). The cells were positive in the K562 cell-killing assay. The cytoplasmic granules of the LGL were isolated, lysed, and the soluble proteins were passed over a Sepharose-anti-human C9 column. The retained protein was eluted with NaCl and found to consist of essentially one component with an M_r of $\sim 70{,}000$ as judged by SDS polyacrylamide gel electrophoresis. The protein was not precipitated by anti-C9 in Ouchterlony tests, but it reacted reproducibly with anti-human C9 by Western blot analysis. By ELISA the cross-reaction with human C9 was approximately 1%. The C9-related lymphocyte protein lacked C9 hemolytic activity, but it formed functional pores in liposomes in the presence of the Ca^{++} (137). These observations have revealed a heretofore unknown immunochemical relationship between the channel-forming protein of cytotoxic lymphocytes and C9 of complement, and they suggest a common evolutionary ancestry of the two proteins. The question, therefore, arises as to whether MAP and C9 are structurally homologous proteins. Cloning and sequencing of MAP cDNA will allow direct comparison with the known structure of C9 (44, 46), which should be accomplished in due time.

The process of target cell killing by lymphocytes as currently understood has been reviewed and summarized by Henkart (132). After binding of the effector to the target cells, the microtubule organizing center and the Golgi apparatus of NK cells and CTL are repositioned to face the contact area with the target cell (133, 134). This rearrangement serves to direct the secretory granules toward the contact site thus allowing granule exocytosis. The MAP along with other substances contained in the granules is secreted into the narrow space between killer and target cell. Under the influence of Ca^{++}, the MAP undergoes conformational rearrangements, insertion into the target membrane and polymerization. Whether the LMAC channel functions merely as a membrane hole or whether it also facilitates the injection of a cytotoxic substance is unknown. Unknown also is how the killer cell protects itself against self-attack and whether metabolically active

nucleated cells are, at least in part, capable of eliminating the killer cell–inserted channels. Although the two molecular mechanisms may be quite different in terms of the reaction pathway and molecules involved, it now appears that the modes of cell killing by complement and by lymphocytes are surprisingly similar.

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CONTENTS

Ann. Rev. Immunol. 1986. 4 : 529–91

THE MOLECULAR GENETICS OF THE T-CELL ANTIGEN RECEPTOR AND T-CELL ANTIGEN RECOGNITION

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INTRODUCTION

T and B lymphocytes participate in the vertebrate immune response through their recognition and elimination of foreign pathogens and macromolecules. Lymphocytes possess both the specificity and the ability to react with a broad range of structures; these properties are mediated through both T-cell antigen receptors and immunoglobulins that serve as antigen receptors for B cells. Despite some similarities, T-cell antigen recognition and function are different in several important respects from the corresponding processes in B cells. First, T cells recognize only antigens that are present on the surfaces of other cells in the context of polymorphic cell-surface molecules encoded by the major histocompatibility complex (MHC) (1–3). The mechanism by which T-cell receptors express an apparently dual specificity for both antigen and polymorphic determinants of the MHC-encoded molecule is not known and has been the subject of much debate. Second, T cells can be subdivided into separate functional categories including cytotoxic effector cells (T_C) (4), inducer or helper cells (T_H) (5, 6), and suppressor cells (T_S) (7). Since immunoglobulins encode both antigen recognition and effector functions within the same molecule, an important question is whether antigen receptors expressed by different T-cell functional classes are distinct. Third, T cells mature in the thymus, an organ composed almost entirely of developing T lymphocytes, while B cells in mammals differentiate in the fetal liver or bone marrow (8–10).

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Development in the thymus is characterized by both rapid proliferation and cell death (reviewed in 11, 12), processes that are probably related to the expression and selection of T-cell receptors. Finally, a number of accessory molecules play an important role in T-cell antigen recognition and/or activation; similar molecules have not been identified for B cells. For example, in humans the T3 molecule, composed of the δ, γ and ε polypeptides, is intimately associated with the T-cell receptor and may serve as an ion channel that functions in T-cell recognition and activation (13, 14). In addition, the human T4 and T8 molecules, and their mouse equivalents, L3T4 and Lyt-2, may bind nonpolymorphic determinants on class-II and class-I MHC molecules, respectively, thereby stabilizing the interaction of T cells with their targets (15–19). Although much has been learned about the functional characteristics of T cells, until recently little was known about the molecular nature of the antigen-specific receptor.

The first breakthrough in the characterization of the T-cell antigen receptor came with the generation of monoclonal antibodies that could bind to only one of a panel of T-cell clones (20–26). These clone-specific antibodies were capable of affecting antigen-specific activation, suggesting that they recognized the antigen receptor. Using these serological reagents, researchers demonstrated that the receptor is composed of a disulfide-linked heterodimer composed of two subunits, denoted α and β, each with a molecular weight of approximately 40–50 kd (kilodaltons). Further characterization of this heterodimer demonstrated that each chain contains portions that are variable and portions that are constant between different T cells; this suggested the presence of both α- and β-chain variable and constant regions (23, 27, 28).

Study of the molecular genetics of the T-cell antigen receptor began when cDNA clones encoding the α and β chains, as well as γ, a third T cell–specific gene family whose function is unknown, were isolated using subtractive or differential hybridization techniques (29–33). In March of 1984, the first papers that characterized cDNA clones encoding the β polypeptide of the T-cell antigen receptor were published (29, 30, 34). Since that date, over 70 papers concerned with the T-cell receptor α and β, and the γ genes of either mice or humans have been written. Progress has been rapid, and in some areas the depth of knowledge of these genes already approaches that we have of the immunoglobulin genes. This article summarizes the current understanding of the organization, rearrangement, ontogeny of expression, and diversification of the genes encoding the α and β chains of T-cell antigen receptor. We discuss the γ genes as well, because of their obvious structural similarity to the α and β T cell–receptor genes. We also attempt to integrate the available knowledge of T cell–receptor genes with theories that account for the mechanisms responsible for MHC-restricted antigen recognition

and immune response gene defects, areas that are still characterized by controversy and speculation.

STRUCTURE AND ORGANIZATION

Structure of the α, β, and γ Chains

The polypeptides encoded by the α, β, and γ genes have significant structural homology to the immunoglobulin genes (29, 31–37). Many of the detailed structural features of these polypeptides have been inferred from the translated sequences of cDNA clones. The α, β, and γ chains are 30–37 kd prior to both glycosylation and cleavage of the leader sequence. They can each be subdivided into seven regions: A hydrophobic leader region of 18–29 amino acids characteristic of all cell-surface and secreted proteins; a variable (V) segment of 88–98 amino acids; a joining (J) segment of approximately 14–21 amino acids; a sequence of 87–113 amino acids that resembles an immunoglobulin constant (C) region; a connecting peptide of varying length; a transmembrane region of approximately 20–24 amino acids; and a small cytoplasmic region of 5–12 amino acids (Figure 1). The V

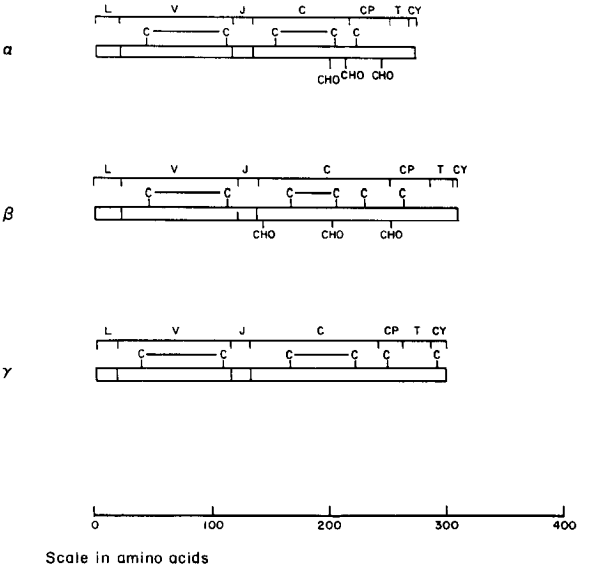

Figure 1 α, β, and γ polypeptides. The murine α, β, and γ chains are represented by open rectangles. Cysteines are represented by a C connected by a vertical line to the rectangle. Potential sites for N-linked glycosylation are represented by -CHO. Above the protein, the relative lengths of seven sections are indicated. L is leader; V, variable segment; J, joining segment; C, constant region; CP, connecting peptide; T, transmembrane region; and CY, cytoplasmic region. The scale is given in amino acids.

and J segments together constitute the V regions that display significant structural similarity with immunoglobulin V regions, including a length of approximately 110 amino acids, a centrally positioned disulfide bridge spanning 63–69 amino acids, and several other conserved amino acids that are believed to be important for protein structure. The α and β chains both have a cysteine, located near the carboxyl terminus in the connecting peptide, that probably participates in the known disulfide bond linking the α and β subunits of the heterodimer. The γ chain has a similar cysteine in the connecting peptide that may participate in a disulfide bond with another γ chain to form a homodimer, or possibly with another polypeptide to form a heterodimer. The β and γ chain have one, and the α chain has two, positively charged amino acids in the transmembrane region. The presence of charged amino acids in this hydrophobic stretch is unusual, although not unprecedented (38). One or more of the positive charges in α and β could form an ionic interaction with a negatively charged aspartic acid in the transmembrane portion of the δ chain of the T3 complex, or possibly with other membrane proteins.

Chromosomal Locations of the α, β, and γ Genes

The chromosomal locations of the murine and human α, β, and γ loci have been determined by a variety of techniques. The results of these studies are summarized in Table 1. In mice, each of the three T-cell gene families that undergo rearrangement is encoded on a separate chromosome. The gene that encodes the murine C_α region is located on chromosome 14, bands C or D (39, 40). A group of related V_α region genes were shown to be linked to the C_α gene (40). In the human α gene family (41), and in the mouse (42, 43) and human (44–46) β gene families as well, all the data are consistent with chromosomal linkage of V and C genes. Thus, as in the three unlinked immunoglobulin gene families, V and C genes that are members of the same gene family, yet separate in the germline (see below), are chromosomally linked.

The genes that encode the C_β regions in mice are located on chromosome

Table 1 Chromosomal locations of the α, β, and γ genes[a]

Gene	Mouse	Human
α	14C–D	14q11–12
β	6B	7q32–35
γ	13A2–3	7p15

[a] Details concerning the experimental methods and references are given in the text.

6, band B (45, 47), which also contains the immunoglobulin κ gene family (48, 49) and the gene encoding the Lyt-2 molecule expressed primarily by MHC class I–specific T cells (50, 51). Genetic analysis using monoclonal antibodies directed against V_β genes in one case (42, 52, 53), or a combination of both monoclonal antibodies and restriction fragment length polymorphisms in another (43), have shown that the β genes are approximately 10–13 centimorgans from the very closely linked Lyt-2 and C_κ genes. The murine γ genes have been located to band A2 or A3 of chromosome 13 (40).

In humans the three T cell–rearranging gene families also are unlinked (Table 1). The α locus is located on chromosome 14, bands q11–12 (41, 54–57). The immunoglobulin heavy-chain genes are also on chromosome 14, but they are located at band q32 (58) and therefore are not closely linked to the α genes. The region of chromosome 14 containing the α genes is often rearranged in T-cell malignancies (59–61). Although there is so far no proof that these rearrangements directly involve either the α gene locus or oncogenes, they may be analogous to the proto-oncogene rearrangements observed in the immunoglobulin loci in B lymphocyte malignancies (62, 63).

The relatively close linkage of the β- and κ-chain genes in mice is not an essential feature of the organization of these gene families. The human β-chain genes have been localized to chromosome 7 (44–46, 64–66) and the human κ genes are found on chromosome 2 (67). Data from in situ hybridization studies indicate that the β genes are located on bands q32–35 of chromosome 7 (46, 65, 66), although in some individuals a significant amount of hybridization is observed on the short arm of chromosome 7 (bands p15–21) (46). In the earliest study in which metaphase chromosome spreads from a single individual were analyzed, most of the observed grains were in fact located around 7p13–21, and relatively few were located near 7q32–35 (45). There is no simple explanation for the variable hybridization of β probes to the short arm of chromosome 7. Although the human γ genes are located at chromosome 7 band p15 (41), the β and γ genes have diverged extensively and should not cross-hybridize. Chromosomal rearrangements with breakpoints that are close to the α, β, and γ gene loci are common in lymphocytes from ataxia telangiectasia patients (68). To a much lesser extent, such abnormalities are also seen in mitogen-stimulated T lymphocytes from normal individuals (69, 70); this suggests that fragile sites in these chromosomes are related to the sites that undergo normal gene rearrangements in T lymphocytes.

Organization of the α, β, and γ Gene Families

Using cDNA clones as probes, researchers have characterized the organization of the α, β, and γ gene families (Figure 2). As in the immunoglobulins,

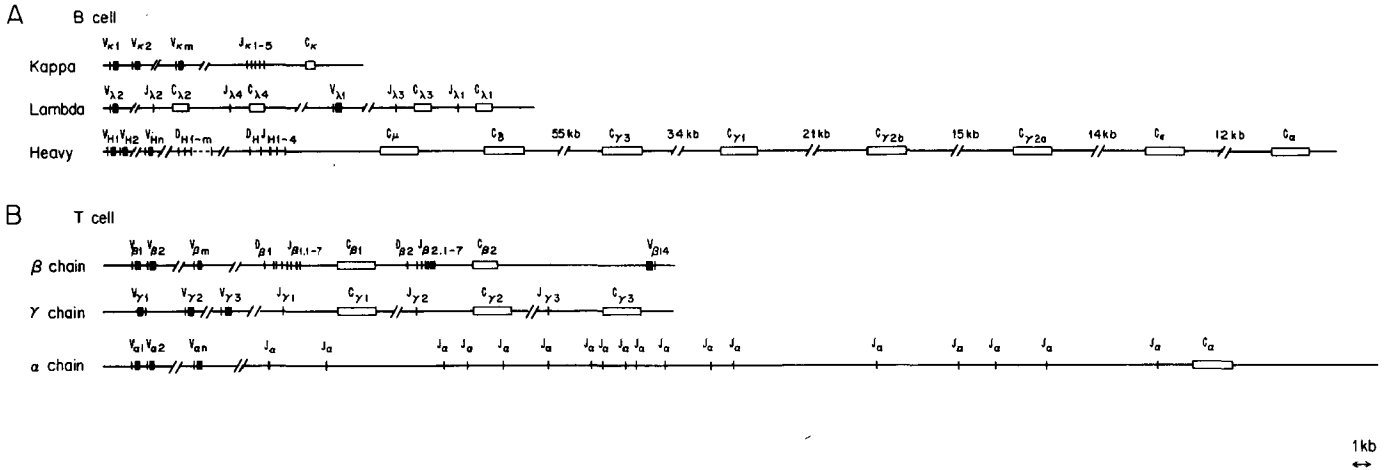

Figure 2 Genomic organization of the six known rearranging gene families of B and T cells. (a) Murine immunoglobulin genes; (b) murine T-cell α, β, and γ genes. Coding sequences are indicated by boxes or short vertical lines; introns and flanking sequences are represented by a thin horizontal line. The separate exons and introns of the different C genes are not shown. A double diagonal line indicates that all the flanking sequences are not depicted. The nomenclature of Davis and colleagues has $C_{\beta 1} = C_T\beta$ and $C_{\beta 2} = C_T\beta'$ (261).

the genes that encode these molecules are actually composed of two parts: a variable gene and a constant gene. The variable gene is composed of either two (V and J) or three (V, D, and J) gene segments (71–76). Each gene family has multiple V and J gene segments and one to three constant genes (71–79). The V, D, and J gene segments are separate in the germline and are brought together by DNA rearrangement during T-lymphocyte differentiation to form the complete V gene (71–76). This DNA rearrangement is mediated by rearrangement signals located directly 3′ to the V gene segments, 5′ to the J gene segments, and on either side of the D gene segments (71–78, 80–82) (Figure 3). The sequences of these rearrangement signals in the three families are similar to one another and to those found in the immunoglobulin gene families. These sequences are composed of a conserved heptamer, 5′ CACAGTG 3′, and a conserved A/T-rich nonamer, separated

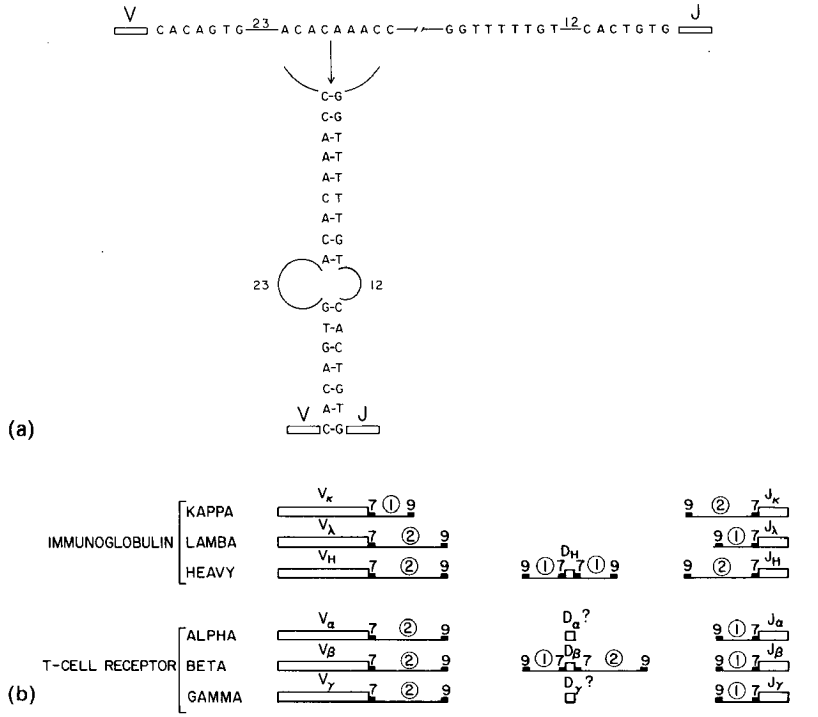

Figure 3 (3a) Rearrangement signals in a hypothetical V-to-J joining. The 23 and 12 refer to the lengths of the nonconserved spacer sequence. The inverse complementarity and the ability of a single strand to form a base-paired stem-loop structure are illustrated. (3b) Rearrangement signals for DNA rearrangement in the six known rearranging gene families, The numbers 7 and 9 represent the conserved heptamer and A/T rich nonamer sequences, respectively. (1) denotes the one-turn or 12-base-pair nonconserved spacer sequence, and (2) denotes the two-turn or 23-base-pair spacer sequence.

by a nonconserved spacer sequence of either 12 or 23 base pairs (Figure 3). The rearrangement signals of any two gene segments that can undergo joining are, with the exception of the spacer sequence, nearly inverse complements of one another, and therefore, these sequences on the same DNA strand can hypothetically base pair with one another (Figure 3a). As in immunoglobulin genes (83–85), DNA rearrangement is believed to occur only when one gene segment has a rearrangement signal with a 12-base-pair spacer (approximately one turn of the DNA helix and therefore denoted a *one-turn signal*) and the other gene segment has a 23-base-pair spacer (approximately two turns of the DNA helix and designated a *two-turn signal*) (Figure 3b).

Variable Gene Segments

The germline variable (V) gene segments of the α, β, and γ gene families are composed of 2 exons separated by an intron of 100–400 base pairs. The first exon is approximately 49 base pairs in length and encodes most of the hydrophobic leader sequence, while the second exon of approximately 300 base pairs encodes the remaining 5 amino acids of the leader and the V segment that comprises the first 88–98 amino acids of the variable-region gene (71–76). All of the α, β, and γ V gene segments have a two-turn signal for DNA rearrangement immediately adjacent to their 3′ ends, as do the immunoglobulin V_H and V_λ gene segments (85) (Figure 3). The rearrangement of a V_β gene segment often deletes some others from the same chromosome, suggesting that many V_β gene segments are located 5′ of both the deleted V_β gene segments and the C_β genes (R. Barth, J. Goverman, unpublished data). However, the murine β locus has V_β gene segments distributed both 5′ and 3′ of the C_β genes. One V_β gene segment is located 10 kb 3′ to $C_{\beta 2}$ in reverse transcriptional orientation (Figure 2) and is utilized by a functional T-cell clone (86). It is not known if other V_β gene segments are located 3′ of the C_β genes. The orientation of the V_α and V_γ gene segments, as well as the V_H and V_L gene segments, to their respective C genes is also unknown.

Figure 4 shows the amino acid sequence of the 16 known murine V_β gene segments. Like the immunoglobulin V gene families, the T-cell antigen-receptor V gene families can be divided into different subfamilies, each consisting of V gene segments more than 75% similar in DNA sequence (87–89). The 16 mouse V_β sequences constitute 14 different subfamilies. We have proposed an arbitrary but simple numerical nomenclature for the V_β gene segment subfamilies and gene segments (88), and it is presented in Figure 4. According to this nomenclature, members of the same subfamily share the first digit and differ in the second; therefore $V_{\beta 8.1}$, $V_{\beta 8.2}$, and $V_{\beta 8.3}$ are all members of the $V_{\beta 8}$ subfamily. The mouse V_β gene family is unique in that

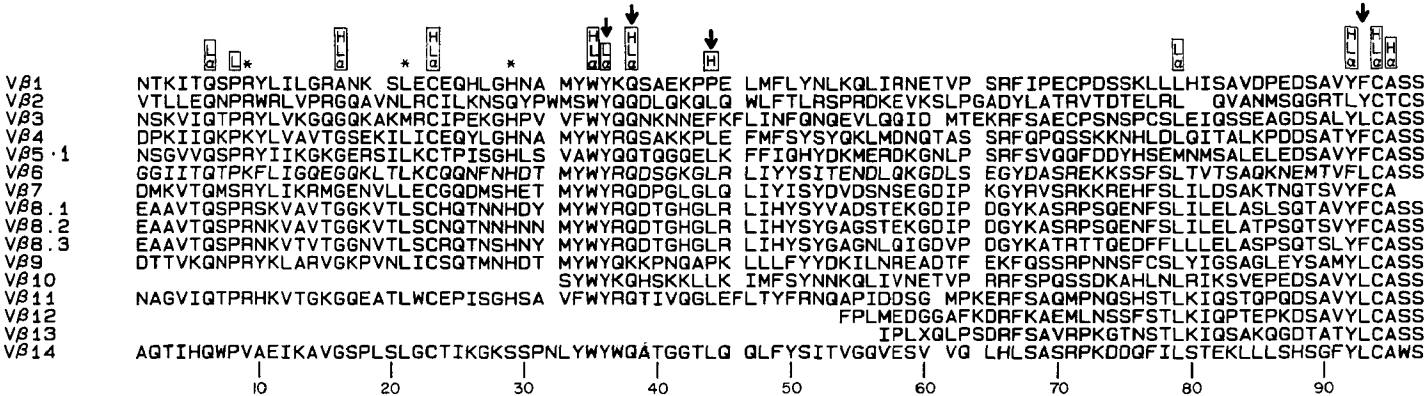

Figure 4 V_β segments. The protein sequences of 16 mouse V_β segments aligned for maximum similarity. The sequence data are derived from the references indicated (31, 32, 34, 86–89). The sequences of $V_{\beta 10}$, $V_{\beta 12}$, and $V_{\beta 13}$ are derived from cDNA clones that do not contain the complete V_β gene segments. A simplified but arbitrary nomenclature is used, equivalent to the following designations from other papers: $V_{\beta 1}$ = 86T1 (34) and $V_{\beta 11}$ (89); $V_{\beta 2}$ = E1 (87) and $V_{\beta 6}$ (89); $V_{\beta 3}$ = 2B4 (32) and $V_{\beta 13}$ (89); $V_{\beta 4} = V_{\beta 9}$ (89), $V_{\beta 5} = V_{\beta 8}$ (89), $V_{\beta 6}$ = LB2 (87) and $V_{\beta 1}$ (89); $V_{\beta 7}$ = HDS11 (31) and $V_{\beta 14}$ (89); $V_{\beta 8.1}$ = C5 (87) and $V_{\beta 12}$ (89). $V_{\beta 8.2} = V_{\beta 4}$ (89); $V_{\beta 9} = V_{\beta 2}$ (89); $V_{\beta 10} = V_{\beta 3}$ (89); $V_{\beta 11} = V_{\beta 5}$ (89); $V_{\beta 12} = V_{\beta 7}$ (89); and $V_{\beta 13} = V_{\beta 10}$ (89). Members of the same subgroup are indicated by a fractional subgroup number, e.g. the $V_{\beta 8.1}$, $V_{\beta 8.2}$, and $V_{\beta 8.3}$ are members of a single $V_{\beta 8}$ subfamily. Conserved amino acids and amino acids important for V_H-V_L interaction are indicated. Conserved positions (>75% frequency of a single amino acid) are indicated by an asterisk if this amino acid is conserved only in the β gene family. If the same amino acid is conserved in other gene families as well, this is indicated by the letters in the open box. L = κ and λ; H = heavy; α = α chain. The arrow indicates invariant or semi-invariant amino acids that are thought to interact with one another to stabilize V_H-V_L interactions in immunoglobulins.

hybridization of Southern blots with V_β gene segment probes has shown that it consists of a large number of single-member gene subfamilies. Of 14 V_β subfamilies, 12 are single copy, and the remaining 2 have only 3 members each (87–89). One of the 3-member subfamilies is $V_{\beta 8}$, and the other is $V_{\beta 5}$, although only 1 of the 3 members of the $V_{\beta 5}$ subfamily has so far been cloned and sequenced. In contrast, the murine V_κ and V_H gene segment subfamilies contain 4 to 50 or more members (90–92). Although the sizes of the V_β subfamilies are small, there are a larger number of known subfamilies than can be found in murine immunoglobulin genes, where only 7 V_H subfamilies and 8 V_κ subfamilies have been identified (90–92). It is not yet known whether human V_β gene segment subfamilies are organized in a way similar to those of the mouse. At present only one human subfamily with 5 members has been characterized (93).

A compilation of the known V_α gene segment sequences is presented in Figure 5 (31, 33, 94, 95). The amino acid sequences shown were translated from the nucleotide sequence of 22 different cDNA clones. The 22 sequences can be grouped into 11 subfamilies, and Southern blots have shown these families consist of 1 to 10 members each (94, 95). The V_α gene family is therefore similar to the V_κ and V_H gene families in that, with one exception, all the V_α gene segment subfamilies have multiple members. A numerical nomenclature for the mouse V_α gene segment subfamilies has been proposed (94), as it was for the V_β genes (Figure 5). The murine V_γ family contains at least one subfamily of 3 members, only 1 of which appears to be utilized frequently (73) (see below). Of the 3 members of this V_γ gene segment subfamily, 2 have been linked and are in opposite transcriptional orientations (73).

Comparisons of the different V_α or V_β gene segments reveal that they can be quite diverse, differing from other gene segments in the same gene family by as much as 70% in DNA sequence and 84% in protein sequence (87–89, 93–95). This is somewhat more diverse than in the immunoglobulin V gene families, where the most divergent V gene segments of the same family differ by as much as 76% in protein sequence (90–92). Despite this diversity, the V_β and V_α genes both have conserved amino acids that are also conserved in immunoglobulin V gene segments (see below).

Diversity Gene Segments

Two murine D_β (diversity) gene segments have been characterized (Figure 2). The more 5′ gene segment, $D_{\beta 1}$, is 12 base pairs long and is identical in sequence in mice and humans (80–82). The more 3′ gene segment, $D_{\beta 2}$, is 14 nucleotides long (82). The sequences of both murine D_β gene segments are G-rich and similar to each other. The D_β gene segments have one-turn recognition signals for DNA rearrangement in their 5′ flanking regions and

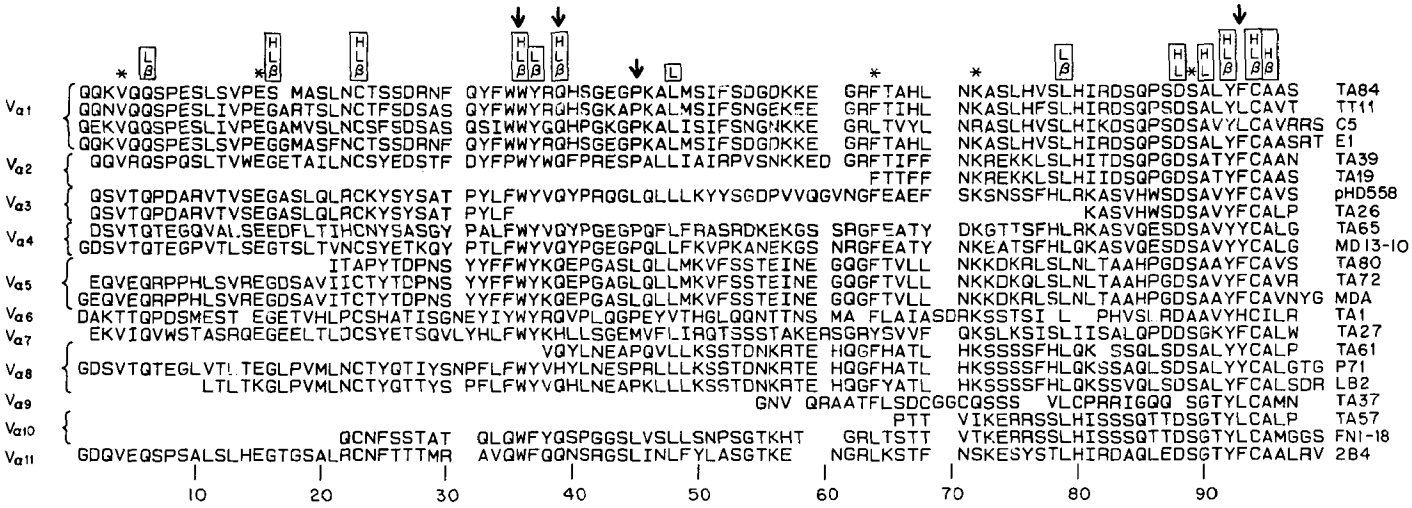

Figure 5 V_α segments. The protein sequences of 22 mouse V_α gene segments aligned for maximum similarity. The sequence data are derived from the references indicated (32, 33, 94, 95). A simplified but arbitrary nomenclature is used as described for the V_β gene segment sequences. Individual V_α gene segments were not numbered, because in some cases it was not clear whether two closely related V_α gene segments were separate members of a subfamily, alleles that differ between mouse strains, or possibly somatic mutants. Conserved amino acid and amino acids important for V_H-V_L interactions are indicated as described in the legend to Figure 4.

two-turn recognition signals in their 3′ flanking region (Figure 3); each is located approximately 500–600 nucleotides upstream to a cluster of J_β gene segments (80–82).

No germline D_α and D_γ gene segments have been isolated, and it is not known whether such gene segments exist. Sequence analysis of both germline and rearranged α and γ gene segments shows that generally all but a few nucleotides in the V-J junctional regions are encoded by the germline gene segments (73–76). These additional nucleotides could be encoded by D gene segments, or they could be the result of other mechanisms that add random nucleotides to the junctional region in the process of joining the gene segments (see below).

Joining Gene Segments

In the mouse there are 14 J_β gene segments, at least 3 J_γ gene segments, and perhaps more than 50 J_α gene segments (71, 73–78) (Figure 2). All of the functional T cell–receptor J gene segments have one-turn recognition signals for DNA rearrangement in their 5′ flanking regions (Figure 3) and a splice-donor signal at their 3′ boundary, which in the primary transcript permits splicing of the joined V-J to the C region sequences. The J_β, J_α, and J_γ gene segments contain 15–17, 19–21, and 19 codons respectively, as well as parts of two other codons at their 5′ and 3′ ends (Figure 6). As in the variable gene segment, the J_α, J_β, and J_γ gene segments encode several

```
                          ↓
                        * ** ** * *
pHDS58      QTGFASALTFGSGTKVIPCLP
TT11       NYGSSGNKLIFGIGTLLSVKP
Jα 80       NTEGADRLTFGKGTQLIIQP
Jα 84       ATSSGQKLVFGQGTILKVYL
Jα 19       VNTGNYKYVFGAGTRLKVIA
Jα 65        NTGYQNFYFGKGTSLTVIP
Jβ 1.1          NTEVFFGKGTRLTVV
Jβ 1.3         SGNTLYFGEGSRLIVV
Jβ 1.5          QPAPLFGEGTRLSVL
Jβ 2.1         NYAEQFFGPGTALTVL
Jβ 2.3         SAETLYFGSGTALTVL
Jβ 2.5         NQDTQYFGPGTRLLVL
Jγ 10.5       SSGFHKVFAEGTKLIVIPS
JH 1           YWYFDVWGAGTTVTVSS
JH 3             WFAYWGQGTLVTVSA
JK 1               WTFGGGTKLEIK
JK 5               LTFGAGTKLELK
Jλ 1               WVFGGGTKLTVL
Jλ 3               FIFGSGTKVTVL
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Figure 6 Comparison of murine J gene segments from six different gene families. In order from top to bottom, six J_α, six J_β, one J_γ, two J_H, two J_κ, and two J_λ gene segments are shown. Conserved amino acids found in most J gene segments are indicated by an asterisk. The position having conserved amino acids important for immunoglobulin V_H-V_L interaction is depicted by an arrow. Data are from the indicated references (71, 73, 75, 77, 78).

conserved amino acids that are also present in immunoglobulin J segments and that are believed to be important for the structure of the immunoglobulin variable regions (see below).

The murine J_β gene segments are grouped into two clusters, $J_{\beta1}$ and $J_{\beta2}$, each containing six functional gene segments and one nonfunctional gene segment (Figure 2). The J_β gene segments in each cluster are separated by 36–421 bp and are located 2–3 kb 5′ to their respective C_β genes (71, 77, 78). Nucleotide sequence analysis of 27 J_α-containing cDNA clones has identified 22 different segments. This implies that the repertoire of J_α gene segments is perhaps larger than 50 functional gene segments and therefore much larger than that of the β locus, which has 12 functional J gene segments, and the murine immunoglobulin gene loci, which each have 3–4 functional J gene segments (94, 95). In addition, the organization of these gene segments is unique in that the mouse and human J_α gene segments are distributed over a large stretch of DNA (74–76). The mouse J_α gene segments span from 3 kb to greater than 63 kb 5′ to the C_α gene (75) (Figure 2). Thus a V_α gene segment that rearranges to one of the J_α gene segments at the 5′ end of the cluster will be expressed on a very large primary transcript that would include a 63 kb intron. Preliminary data indicate the J_α gene segments are also further apart from one another than either the J_β, J_H, or J_κ gene segments, as sequence analyses indicate that no two J_α gene segments studied to date are closer to one another than 500 base pairs (74–76). There are at least three murine J_γ gene segments. These are not organized in a J gene segment cluster; instead, each one is linked 3–4 kb 5′ to a different C_γ gene (73). Therefore the J_γ-C_γ gene organization is similar to that of the immunoglobulin λ genes (Figure 2).

Constant Region Genes

C_β GENES In mice and humans there are two C_β genes, denoted $C_{\beta1}$ and $C_{\beta2}$ (77, 78, 96–99). The β locus is a tandem duplication of DNA containing one D_β gene segment, a cluster of seven J_β gene segments, and a C_β gene (Figure 2). The two C_β genes are very similar to each other. The two mouse C_β proteins indeed differ by four amino acids; in humans, they differ by six. In both species the differences are concentrated towards the 3′ end of the gene (77, 78, 97, 99).

The murine and human C_β genes are encoded by four exons that, in contrast to the immunoglobulin C genes, do not correlate with the presumed functional domains of the constant region (77, 78, 99). In the mouse, the first exon is 375 base pairs in length and encodes a block of 113 amino acids with homology to immunoglobulin constant regions followed by the first 12 amino acids of the connecting peptide. This is the only instance in which a block of sequence homologous to an immunoglobulin V or C region is not encoded in a separate exon (see below). The 113-amino-

acid sequence contains two cysteines that could form a disulfide bond spanning 60 amino acids, and it is most homologous to the C_λ genes (37%) and the first domain of the immunoglobulin γ heavy chain constant regions (32–36%) (78). The remainder of the connecting peptide is encoded by a second exon of 18 nucleotides and a portion of the third exon of 107 base pairs. In immunoglobulin heavy chain constant regions, the second exon encodes a proline-rich hinge region that permits flexibility in the constant region (100–105). Although the second exon of the C_β gene is positioned in approximately the same place as is the heavy chain hinge exon, it does not encode any prolines and thus does not resemble a hinge region. The remainder of the third exon encodes most of the transmembrane region, which consists of neutral and nonpolar amino acids, with the exception of a single lysine. The fourth exon of 179 base pairs encodes 1 amino acid of the transmembrane region, a 5–6 amino acid cytoplasmic region, and the 3′ nontranslated region.

C_α GENE There appears to be only one C_α gene in both mice and humans (37, 74–76). Like the C_β genes, the C_α gene consists of four exons. In humans, the first exon is 261 bp long and encodes the region that is homologous to immunoglobulin constant regions. However, in the C_α gene, this region is only 87, rather than 113, amino acids long, and it contains a cystine bridge spanning only 49 amino acids, as opposed to the typical 60–75 amino acids. The second exon is 45 bp long and encodes 15 amino acids of the connecting peptide. The third exon, 108 bp, encodes the remainder of the connecting peptide, the transmembrane region, and a short cytoplasmic region, while the fourth exon encodes a 3′ nontranslated region of 558 bp (76).

C_γ GENES There are three murine C_γ genes (73). One of these C_γ genes is a pseudogene in that mutation in a donor splice recognition signal would probably prevent successful splicing of a C_γ transcript. In humans, there are two C_γ genes that are separated by approximately 6 kb of DNA (106). Unlike the C_α and C_β genes, the murine C_γ gene has only three exons (73). The first exon, 330 bp long, encodes the region with homology to immunoglobulin constant regions; the second, 30 bp long, encodes a portion of the connecting peptide; the third, 545 bp long, encodes the remainder of the connecting peptide, the transmembrane sequence, a 12 amino acid cytoplasmic region, and the 3′ nontranslated region.

EVOLUTION

Immunoglobulin Gene Superfamily

A gene superfamily is a set of multigene families and single copy genes related by sequence (implying a common ancestry), but not necessarily

related in function (107, 108). The immunoglobulin and T cell–rearranging gene families are members of the immunoglobulin gene superfamily—named after the first well-studied members of this family (109). Over the past five years, gene cloning and nucleotide sequence analyses have led to the identification of a number of other genes that are considered members of this superfamily because of their sequence similarity with immunoglobulins. Therefore, the immunoglobulin gene superfamily now includes: the three B-cell and the three T-cell rearranging gene families; the MHC-encoded class-I heavy-chain (110, 111) and class-II (112, 113) genes; a number of single copy genes including Thy-1 (108, 114) and MRC-OX2 (115), antigens of unknown function that are expressed in brain, in thymus, and on other cell types; the poly-Ig receptor, which transports polymeric IgA and IgM immunoglobulin across mucous membranes (116); the T-cell accessory molecules T4 and T8 (116a, 117, 118); and β_2-microglobulin that is expressed in association with the heavy chain of the MHC class-I molecule (119) (Figure 7).

Immunoglobulin Homology Unit

The polypeptide members of this gene superfamily are all constructed of one or more immunoglobulin homology units. Each homology unit is approximately 110 amino acids long and has several conserved amino acids and a centrally placed cystine disulfide bridge usually spanning 60–75 amino acids. From sequence analyses, it can be predicted that the immunoglobulin homology units all probably form a conserved tertiary structure denoted the *antibody fold*, composed of two sheets of 3–4 antiparallel β-pleated strands (120). Pairs of homology units can fold together in turn to create discrete polypeptide domains (e.g. V_L-V_H or C_L-C_H) characteristic of immunoglobulins. Thus, the tertiary structure of one homology unit appears to facilitate interactions with a second homology unit to form a functional domain. At the DNA level, nearly every homology unit is encoded by a separate exon; this demonstrates a correlation between the distinct structural features of these proteins and the exon-intron structure of the corresponding genes.

Genealogical Tree

A genealogic tree has been constructed for the immunoglobulin gene superfamily (109) by assuming that evolutionary relatedness correlates with both the degree of sequence similarity among the members and with other features such as intron-exon structure and the ability to undergo DNA rearrangements (Figure 7). The figure illustrates the hypothesis that many of the molecules important for the vertebrate immune response are encoded by genes that descended from a single ancestral sequence. According to this

analysis, one of the earliest events in the evolution of the gene superfamily was a duplication leading to the divergence of V and C exons. Contemporary V and C homology units have little primary sequence similarity, suggesting an ancient divergence, although in immunoglobulins they retain similar tertiary structures. V and C homology units can be

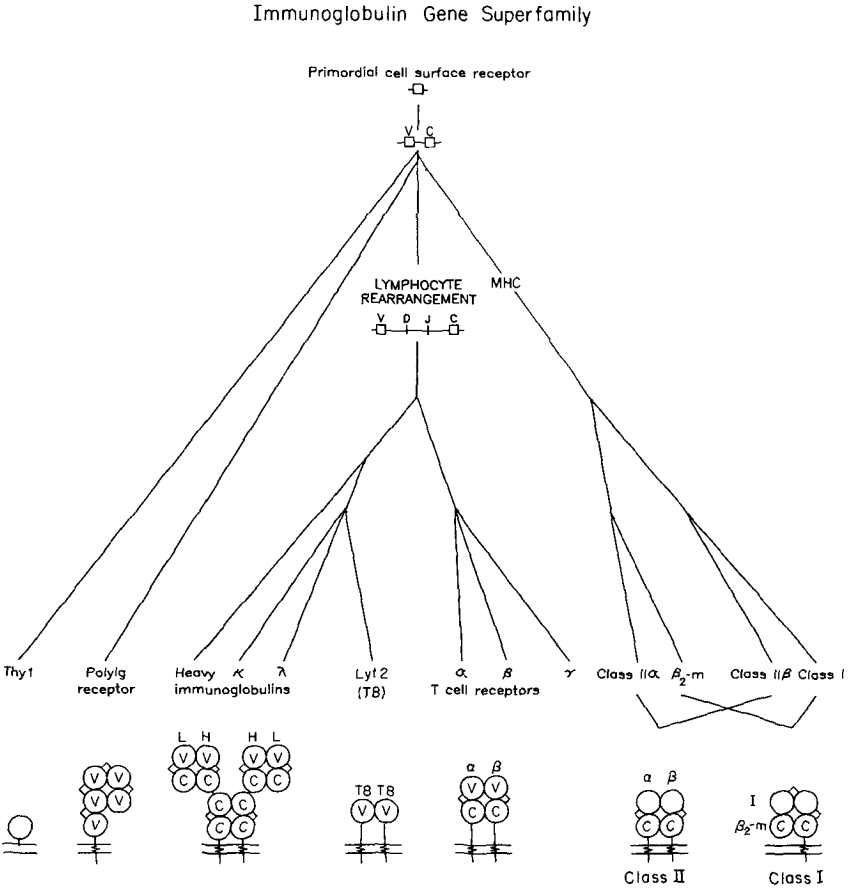

Figure 7 A schematic representation of the evolution of the immunoglobulin gene superfamily. V and C denote a V- or C-like homology unit, respectively. The open circles for the MHC molecules do not exhibit significant sequence similarity with the immunoglobulin homology units, although they are of similar length. The Thy-1 homology unit does exhibit sequence similarity, although it is not easily classified as V or C and may have diverged prior to the V-C divergence. The horizontally paired homology units represent probable domain structures, apart from those speculated for the poly Ig receptor and the T8 molecule. The T4 gene and MRC-OX2 (not shown) each have both a single V and a single C homology unit.

distinguished from one another by their length and by the presence of certain V- or C-specific conserved amino acids. Members of the immunoglobulin gene superfamily are composed of different numbers of either V and/or C homology units. A second critical early event was the acquisition of the ability to rearrange DNA, which may have arisen from the capture of a complex transposon by a primordial V gene (121, 121a).

REARRANGEMENT AND EXPRESSION OF α, β, AND γ GENES

A large panel of lymphoid cells has been tested for rearrangement and expression of T-cell receptor α, β, and γ genes. Several questions have been addressed by these analyses. First, in light of the dual specificity of T lymphocytes for antigen and self-MHC-encoded molecules, is it possible that two T-cell antigen receptor molecules are expressed on individual T cells? This could occur if other receptor gene families besides α and β are expressed, or if the expression of the V genes in at least one of the defined receptor gene families is not subject to allelic exclusion. Second, are there antigen receptor isotypes that are associated with the different functional categories of T lymphocytes? Finally, are gene segment rearrangements regulated so that they occur in a well-defined progression, and, if so, what insight does it give us into the ontogeny of T lymphocytes and into possible mechanisms leading to allelic exclusion?

Tissue-Specific DNA Rearrangements

The available data summarized above indicate that the rearrangement processes in the immunoglobulin and T cell–specific gene families are quite similar. For example, similar recognition sequences for DNA rearrangement are used in all six gene families (Figure 3). In spite of these similarities, the complete rearrangement of T-cell antigen receptor and immunoglobulin genes appears to be tissue specific. Approximately 10% of mouse T lymphocytes have D_H-J_H rearrangements (122–126), but V_H (127–130) and light chain gene segments do not rearrange in these cells (122, 131). Similarly, only 6 cell lines out of a sample of more than 100 B lymphocyte tumors and hybridomas that were examined have undergone β gene rearrangement (F. Alt, M. Kronenberg, R. Perlmutter, unpublished observations; and 132–134). No evidence indicates that these events lead to the production of a functional β polypeptide. Insight into this tissue-specific regulation has emerged from studies in which DNA constructs containing unjoined D_β and J_β gene segments were introduced into B-cell lines that carry out immunoglobulin gene segment rearrangements (G. Yancopoulos, K. Blackwell, L. Hood, F. Alt, unpublished data). In these cells, the β gene

segments are rearranged in a site-specific fashion as efficiently as D_H and J_H gene segments introduced in a similar manner. These data suggest that the DNA recombinational machinery in B and T cells is very similar and that other cell type–specific features such as chromatin structure may determine which gene families rearrange in the two cell types.

Mechanisms of DNA Rearrangement

Several mechanisms have been proposed to account for immunoglobulin gene segment rearrangements (Figure 8). The DNA between gene segments may loop out; the stem of this loop may be formed by base pairing between the recognition signals for DNA rearrangement (Figure 3a). The stem-loop structure may then be excised to complete gene segment joining. Most of the rearrangements in the immunoglobulin heavy chain gene family result in the deletion of the DNA sequences between the joined gene segments from the genome and are therefore consistent with the deletion model (135). However, κ gene rearrangements often appear incompatible with this deletion model (136–141), and consequently three other models for rearrangement have been proposed: homologous but unequal sister chromatid exchange (137, 138, 140), inversion (139, 142, 143), and reintegration of the deleted sequences (136, 137) (Figure 8).

Several of these models are required to explain the rearrangements that occur in the β gene family. Although the majority of T cells (24/37) analyzed in detail had a β gene rearrangement pattern that is consistent with the deletion model, 13 out of 37 T cells had Southern blot patterns that could not be explained in this way (144). In some cases, the DNA in the region between joined gene segments was retained in the genome, an observation consistent with any one of the three additional models (144). In other cases, a partial duplication of the J_β-C_β locus was observed, an observation consistent only with homologous but unequal sister chromatid exchange (144, 145).

The detailed analysis of all the β gene segment rearrangements in one T_H cell line has shown that the V_β gene segment expressed in this cell had rearranged by inversion (86). As noted above, in germline DNA one of the V_β gene segments, $V_{\beta 14}$, is located 10 kb 3′ to the $C_{\beta 2}$ gene and in the opposite transcriptional orientation to the J_β gene segments and C_β genes (Figure 2). Two different mechanisms were required to form the complete V_β gene in this cell (Figure 9). First, the $D_{\beta 1}$ gene segment joined to the $J_{\beta 2.3}$ gene segment via a deletion or homologous but unequal sister-chromatid exchange. The $V_{\beta 14}$ gene segment then rearranged, inverting a 15 kb sequence of DNA. One inversion breakpoint contains the joined V_β-D_β-J_β gene, while the other encompasses the reciprocal recombination product containing the heptamer 3′ of the V_β gene segment joined to the heptamer 5′

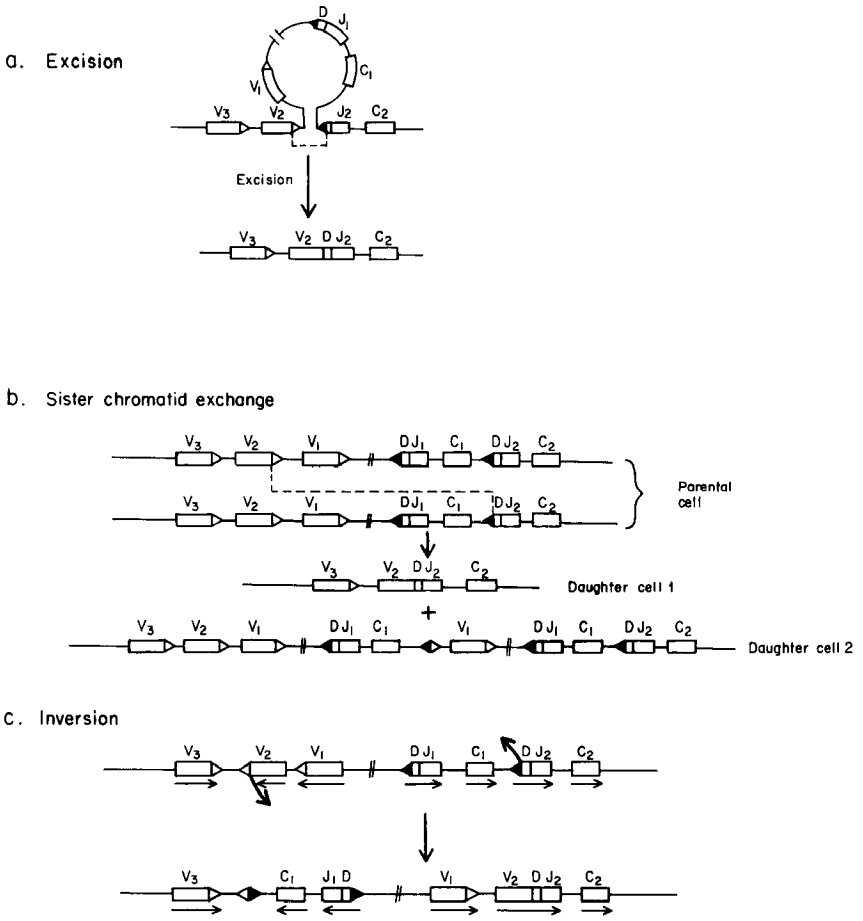

Figure 8 Three models for antigen receptor gene rearrangement. ◀ = one-turn recognition signals for DNA rearrangement. ▷ = two-turn recognition signals for DNA rearrangement. For clarity, only rearrangement of a hypothetical V2 gene segment to an already-joined D-J2 gene segment is illustrated, although the same models apply to D-J rearrangements. (a) Excision or deletion. A stem and loop is formed by base-paired DNA-joining recognition sequences, this stem-loop is the excised, and the adjacent sequences, connected by a broken line, are joined. (b) Unequal sister chromatid exchange. The right-hand brackets represent the connection of the sister chromatids at the centromere. The broken line connects the breakpoints in the two chromatids. The diamond on the horizontal line between C1 and V1 denotes a recombination breakpoint containing DNA rearrangement signals joined in a head-to-head fashion. (c) Inversion. The horizontal arrows indicate the transcriptional orientation of the various gene segments and C genes. This figure is adapted from reference 144.

of the $D_{\beta 1}$ gene segment (86). Thus, taking into account all the data, it appears that all three of the mechanisms depicted in Figure 9 may be employed for β gene rearrangements.

The detailed mechanisms of rearrangement in the α and γ gene families have not been so well characterized. It is also not clear why rearrangements in some of these gene families are always characterized by deletions, such as are observed for the immunoglobulin heavy chain genes, while in others, such as the κ and β gene families, different mechanisms also appear to operate. Any V gene segment, such as $V_{\beta 14}$, that is in the opposite orientation to the J gene segments must undergo an inversion in order to be expressed. Perhaps an inversion of germline DNA that included some V gene segments occurred during the evolution of both κ and β gene families; therefore, they require a second inversion as a mechanism for their somatic rearrangement and expression. The factors that might facilitate unequal sister chromatid exchanges in some but not other gene families, however, are unclear.

Allelic Exclusion

The rearrangement of β genes in T cells normally occurs on both chromosomal homologues. In a panel of 37 clonal T lymphocyte lines, only 3% of the $J_{\beta 1}$ gene segment clusters and 13% of the $J_{\beta 2}$ gene segment clusters

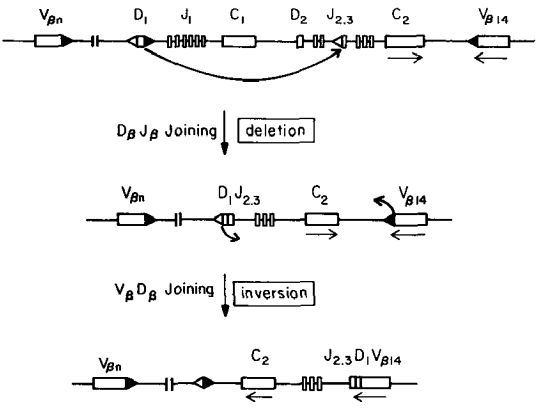

Figure 9 β gene rearrangement by two mechanisms in a T-helper-cell line (86). The drawing is not to scale. ◁ and ▶ represent one-turn and two-turn DNA rearrangement signals, respectively. For simplicity, only some of the rearrangement signals adjacent to the gene segments are shown ◁▶ represents the joined rearrangement signals that result from the inversion, and the horizontal arrows indicate the transcriptional orientation of some of the relevant coding sequences.

were in a germline configuration (144). Densitometric analysis of Southern blots from heterogeneous T-cell populations demonstrates that normal T cells also display a similar degree of β gene rearrangement (146, 147).

Southern blot analysis of three T-cell lines and the isolation of all the β gene rearrangements from three others indicate that each of these six T cells can express only a single V_β gene (86, 144, 148). Therefore, although β gene rearrangement occurs on both chromosomal homologues, the expression of these genes may be allelically excluded in that only one allele is productively rearranged and used in the synthesis of a functional β chain in individual T cells. Whether a β gene rearrangement is productive is determined by the joining of the V_β gene segment, which sets the translational reading frame of the J_β gene segments. Because only one of the three possible translational reading frames is productive for each J_β gene segment, and because the addition and deletion of nucleotides at the V_β-D_β junction is apparently random, in two thirds of the V_β-D_β-J_β rearrangements the J gene segments will not be in the proper translational reading frame.

The α and γ gene rearrangements also generate diversity through the random deletion and possible addition of nucleotides at the junction between joined gene segments (73–75, 149) (see below). As a consequence, many rearranged genes in these families should not be in the proper translational reading frame. There is not yet enough information to determine whether α and γ gene rearrangements are allelically excluded, although this is a reasonable hypothesis. Southern blots of four cytotoxic T-cell clones initially analyzed showed they had undergone γ chain gene rearrangements on both homologues (31); however, one of these T-cell clones, analyzed in more detail, had one productive and one nonproductive γ gene rearrangement (149).

Beta Gene Rearrangements in Different Cell Types

HELPER AND CYTOTOXIC T CELLS AND T-CELL TUMORS β gene rearrangement is nearly ubiquitous in human and murine T cells. In humans, 4/4 helper T-cell lines, 7/7 cytotoxic T-cell lines, 1 cell line with both cytotoxic and helper function (150, 151), and 65/71 T-cell tumors all exhibited β gene rearrangement (132–134, 152). The remaining 6 tumors had germline β genes, and they may be similar to immature T cells, or they may not in fact belong to the T-cell lineage. Similarly, in the mouse, 28/28 helper-T-cell lines and hybridomas, 16/16 cytotoxic-T-cell lines, and 14/14 T-cell tumors exhibited β gene rearrangements (144, 153). Therefore, all cytotoxic and β helper cells rearrange and probably express β genes, although in many cases there is no conclusive evidence that these rearrangements are productive.

SUPPRESSOR T CELLS Suppressor T cells are defined by a number of different and complex immunological assays. Of 15 mouse suppressor-T-cell hybridomas tested, 2 exhibited β gene rearrangements, 1 had germline β genes, and the remaining 12 hybrid cells had apparently deleted the β gene loci from both chromosomes that were contributed by the suppressor-T-cell fusion partner (144, 153). Therefore, the T_S cells in these cases do not utilize the β chain in their antigen receptors. In contrast, 5/5 human suppressor-T-cell lines tested exhibit β gene rearrangements, and evidence indicates that some of these cells express a typical α/β heterodimer (150, 151, 154). These data imply that there may be several classes of T_S cells, only some of which utilize the β genes, although other explanations are possible.

NATURAL KILLER CELLS Lymphoid cells that spontaneously exhibit cytotoxic activity against a variety of tumor and nontransformed cell types are known as natural killer (NK) cells. NK cells can have several β gene phenotypes. Cloned murine NK lines that are cultured in the presence of IL-2 have β gene rearrangements and transcripts (155), although several rat large granular lymphomas that display NK activity have germline β genes (156). Peripheral blood cells from normal human donors that express cell-surface markers characteristic of NK cells also had β gene rearrangements, although there was significantly less rearrangement than was observed in T-cell populations (147). This result is consistent with the results of an analysis of a panel of human clones with NK activity; those that express T3 have β gene rearrangements and α transcripts, while those that do not express T3 have no α transcripts and may only have D_β-J_β rearrangements (157). Collectively these results indicate that NK cells are a mixed population with regard to β gene rearrangement and that expression of an α/β heterodimer is not required for NK activity. It remains possible, however, that in some cases T-cell antigen receptors participate in the recognition of NK target molecules.

Transcription of Beta Genes

There are two predominant size classes for the β transcript: a 1.3 kb RNA that contains a V_β gene and a 1.0 kb RNA that does not (82, 144, 150). The 1.0 kb transcripts appear to be derived from rearrangements that joined a D_β to a J_β gene segment in the absence of a V_β gene segment rearrangement (82). In the mouse, both $D_{\beta 1}$ and $D_{\beta 2}$ gene segments have been identified in cDNA clones that contain D_β joined to J_β gene segments but that lack V_β gene segments; this implies that promoters exist in the 5′ flanking regions of both germline D_β gene segments (81, 82). Because the transcribed 5′ flanking regions also contain open-reading frames and an in-frame methionine

codon that could serve as a start-signal for translation (82), the sequences of these rearranged D_β-J_β genes could encode truncated polypeptides containing sequences 5′ to the D_β gene segments, as well as a D_β, J_β, and C_β sequences. Although so far no evidence exists for these truncated β polypeptides, in immunoglobulins the analogous D_H-J_H rearrangements do give rise to both transcripts and truncated polypeptides containing the C_μ gene product (158). Other transcripts that may be derived from unrearranged genes and several aberrantly spliced β transcripts have also been observed (R. Barth, unpublished observations, and 159).

Alpha Gene Rearrangement and Transcription

The C_α gene is transcribed in a variety of cell types including helpers, cytotoxic cells, many tumors, and the $T3^+$ NK clones mentioned above. Two size classes of α chain RNA have been described—a 1.7 kb transcript that contains V_α sequences and a 1.4 kb transcript (32, 33). By analogy with the β chain genes, the shorter transcript could be derived from a D_α-J_α rearrangement or from a J_α-C_α transcript initiated by a promoter 5′ to the J_α gene segment. Consistent with the latter hypothesis is the identification of a cDNA encoding a germline J_α-C_α transcript (A. Winoto, unpublished). The levels of α and β RNA in peripheral T cells are roughly equivalent (32, 146, 160). However, the thymus contains far more β than α RNA, and this is consistent with the hypothesis that the β genes rearrange and are expressed before the α genes (32, 146) (see below).

Gamma Gene Rearrangement and Transcription

Transcripts containing γ gene sequences were found in 4/4 cytotoxic cells tested, but in only 1/10 helper-T-cell lines and hybridomas (31, 149), and in none of the human NK clones tested (157). This pattern of expression suggests that the γ chain and MHC class-I recognition or antigen-specific T-cell cytotoxicity are in some way linked. Compared to β transcripts, γ RNA is at least 10-fold less abundant in both adult thymocytes and peripheral T cells (32, 146, 160).

While transcription of γ gene is confined primarily to cytotoxic cells, γ gene rearrangement is equally prevalent in both helper- and cytotoxic-T-cell populations. DNA prepared from $Lyt\text{-}2^+$ cells that are predominantly cytotoxic and suppressor T cells, and DNA prepared from $L3T4^+$ cells that are predominantly helper T cells, had approximately the same amount of γ gene rearrangement (149). In addition, 8/10 IL-2-producing T hybridomas restricted by class-II-MHC molecules have γ gene rearrangements and no germline γ chromosomes (R. Haars, J. Kobori, N. Shastri, unpublished data; 149). The other two hybrid cells deleted the γ genes that were not

derived from the BW5147 fusion partner. All of the $T4^+$, $T8^-$ human cell lines tested also had γ gene rearrangement (106). It is not yet known whether any of these rearrangements are productive.

All or most of the γ gene rearrangements in murine T cells are similar; this suggests that murine V_γ and J_γ gene segment diversity is quite limited (146, 149). In the human, most cell lines have different rearrangements, suggesting that V_γ gene segments may be more diverse (106).

T-Cell Receptor Isotypes

Isotypes are defined as multiple nonallelic forms of constant-region genes. The immunoglobulin heavy chain gene locus is characterized by several C region isotypes that participate in different effector functions (reviewed in 161). There is no evidence for C_α isotypes; only one C_α gene hybridizes with the available probes (37, 74, 75), and this C_α is transcribed in a variety of cell types (32, 33). The $C_{\beta 1}$ and $C_{\beta 2}$ genes are isotypes, yet there is no evidence that expression of either one of these two C_β genes is related to the ontological development of a T cell (146, 160), its function, or the specificity for either antigen or MHC molecule. For example, both helper and cytotoxic T cells can express either the $C_{\beta 1}$ or the $C_{\beta 2}$ genes (144). The $C_{\beta 2}$ transcript is more abundant than $C_{\beta 1}$ in RNA from lymphoid tissues (146), and T-cell clones use the $C_{\beta 2}$ gene more often than the $C_{\beta 1}$ gene (88, 144). However, this bias probably reflects an inherent statistical bias for rearrangement of D_β gene segments to the $J_{\beta 2}$ gene cluster (see below). Finally, the functional equivalence of $C_{\beta 1}$ and $C_{\beta 2}$ is best demonstrated by NZW mice that lack a $C_{\beta 1}$ gene, as well as $D_{\beta 2}$ and $J_{\beta 2}$ gene segments, and that have apparently normal T-cell function (162). Since it appears that all helper and cytotoxic and some suppressor T cells employ an α/β heterodimer, the T-cell antigen receptor apparently lacks the equivalents of both functional heavy chain isotypes and the immunoglobulin heavy chain class switch.

Unlike expression of the α and β genes, the pattern of γ gene transcription suggests that expression of this molecule, possibly in association with some other cell-surface protein, is related to either class-I MHC recognition or cytotoxic function. Analysis of γ gene expression in MHC class II–specific cytotoxic T cells should establish whether the type of MHC molecule recognized or the T-cell function correlates better with expression of this molecule. The lack of γ gene transcripts in some cell types, however, raises the possibility of an isotypic γ chain functional equivalent that could be expressed by these cells.

THE ONTOGENY OF T-CELL ANTIGEN RECEPTOR REARRANGEMENT AND EXPRESSION

The ontogeny of α, β, and γ gene rearrangement and expression in fetal and adult cells has been studied by several groups. Two major issues have been addressed by these studies. First, do rearrangements occur in a sequence of steps, as is the case in the three immunoglobulin gene families? If rearrangements occur in this manner, it might be possible to assess unambiguously the developmental stage of some T cells by determining which gene families had and which had not rearranged. Second, when and where in development are these genes first expressed?

Ordered Rearrangement and Expression

ORDERED EXPRESSION OF IMMUNOGLOBULINS The study of the developmental control of T-cell antigen receptor and γ gene rearrangement and expression has been influenced by studies on the ontogeny of immunoglobulin rearrangement and expression in B cells. Based on these immunoglobulin gene studies, a regulated model for the rearrangement of gene segments in B cells has been proposed that accounts for the observed rearrangements in developing B cells and suggests a mechanism for allelic exclusion. Immunoglobulin rearrangement and expression can be divided into four separate stages (reviewed in 163). The first stage is D_H-J_H rearrangement (135, 164), and the second stage involves the rearrangement of the V_H gene segment to the joined D_H-J_H gene segments. According to the regulated model, if this second rearrangement event is nonproductive, further gene segment rearrangements occur on the other chromosomal homologue (135). A productive V_H-D_H-J_H rearrangement is believed to inhibit further heavy chain gene rearrangements; it has been proposed that the synthesis of a heavy chain polypeptide plays a role in this process (165). The third step involves the rearrangement of the V_κ and J_κ gene segments (166). Because this appears to require productive heavy chain gene rearrangement, the heavy chain polypeptide may also play a role in activating gene rearrangement in the κ gene family. The successful rearrangement and expression of a κ protein and the subsequent formation of a complete cell-surface immunoglobulin molecule is believed to be involved in the termination of further κ rearrangements (167, 168). The fourth step is the rearrangement of the λ light chain genes, which only occurs if the κ genes have rearranged nonproductively on both homologues (167, 169).

FETAL TISSUES AND T CELLS To determine if there is an order to the rearrangement and transcription of the α, β, and γ genes, RNA and DNA

from the appropriate fetal tissues have been hybridized with DNA probes. The results of these studies are summarized in Table 2. The mouse has a gestation period of approximately 20 days, and immunocompetent thymocytes can be detected shortly before birth (170). The thymus itself develops from nonlymphoid cells in the pharyngeal pouch and cleft, and other tissues, and is first seeded by lymphocytes around the eleventh day of gestation (171). Most of the prethymic cells originate in the fetal liver. The thymus increases dramatically in cell number between day 12 and day 18, due to both cell migration from fetal liver and proliferation of thymocytes.

Analysis of the transcripts present in fetal tissues indicates that γ RNA appears first, followed by β and finally α, and that the level of γ RNA decreases around the time that the α message appears (146, 160, 172, 173) (Table 2). Although no transcripts were detected in fetal liver from any stage, γ RNA could be detected in day-14 fetal thymus, the earliest day tested (146). At this stage, the thymus contains mostly developing T cells. The Lyt-2 and L3T4 molecules are not yet expressed (174–176), but most day-14 fetal thymocytes express the Thy-1 antigen (175, 176), and some express IL-2 receptors (177, 178). No C_α RNA can be detected at this stage (146, 160, 173). A 1.0 kb transcript derived from D_β-J_β rearrangements is

Table 2 Gene rearrangement and expression in developing T lymphocytes[a]

	DNA rearrangements			RNA transcripts		
	γ	β		C_γ	C_β	C_α
		D-J	V-D-J		(1.3 kb)	(1.7 kb)
Fetal liver	+/−	−	−	−	−	−
d12–19	(d12–13 only)					
Fetal thymus						
d14	+/−	+/−	−	+	−	−
d15	+/−	+	+/−	+	+/−	−
d16	+	+	+	+	+	+/−
d17	+	+	+	+	+	+
Neonatal thymus	+	+	+	+	+	+
Adult thymus						
Lyt-2$^-$, L3T4$^-$	nd	+	+	+	+	+/−
Lyt-2$^-$, L3T4$^-$, Pgp-1$^+$	nd	−	−	nd	nd	nd

[a] The table is compiled from the references cited in the text. Information on other adult thymus subpopulations can be found in the indicated references (12, 173, 192, 193). "+" indicates rearrangement or transcript is present. For detectable transcripts, different levels of abundance are not distinguished. "+/−" indicates rearrangement is present but in less than 10% of the chromosomes, or the transcript is barely detectable. "−" indicates rearrangement or transcripts not detectable. nd indicates not done. The presence or absence of the complete 1.3 kb C_β and 1.7 kb C_α transcripts are listed in the table; the shorter C_β and C_α transcripts are not included.

present, but 1.3 kb transcripts containing V_β genes can first be barely detected on day 15 (146, 172). On day 16, a relatively large amount of 1.3 kb β gene RNA is present (146, 160, 172), and α gene transcripts can be detected, although most of the α RNA is found in the shorter 1.4 kb RNA transcript (146). Substantial levels of both α and β RNA are present by day 17 (146, 160, 172, 173). The presence of significant amounts of complete α and β gene transcripts on day 17 is consistent with biochemical and immunochemical studies demonstrating that cell-surface expression of α/β heterodimers can first be detected on this day (172, 179).

Analysis of DNA prepared from fetal organs or from hybridomas made by fusing fetal cells with the BW5147 T lymphoma indicates that the order of rearrangement of the β and γ genes in fetal tissues is consistent with the order of expression: namely, V_γ joins to a J_γ gene segment; around the same time or soon after, D_β-J_β gene segment joining occurs, followed by V_β rearrangement (146, 180).

IMMATURE ADULT THYMOCYTES Experiments on selected populations of adult thymocytes have yielded results similar to those obtained with fetal thymocytes of day 14–16 gestation. The Lyt-2$^-$L3T4$^-$, or double-negative, subpopulation resembles fetal thymocytes from days 14–15 gestation in that both populations do not express the Lyt-2 and L3T4 antigens, both are rapidly dividing, and both probably rearrange and express the α, β, and γ genes in a similar order (160, 181, 182) (Table 2). These double-negative cells, which are thought to give rise to the other thymus subpopulations (reviewed in 183, 184), have large amounts of γ (160) and very little, if any, α RNA (181, 185). In addition, although most of the cells in the adult thymus have already undergone β gene rearrangements on both homologues, some of the double-negative cells have germline β genes (181, 182). A subpopulation of the double-negative cells express the cell-surface antigen Pgp-1, which is also found on bone marrow cells and on many day-14–15 fetal thymocytes. This subpopulation, which may represent the earliest lineage in the adult thymus, has almost no β gene rearrangement (182). Therefore, despite the fact that the different immature subpopulations are a minute fraction of the adult thymus and are difficult to separate from one another, the γ-β-α progression also appears to occur in these cells.

In summary, although it is still controversial whether a single differentiation pathway characterizes T-cell development, or whether cortical and medullary cells differentiate separately (11, 12, 184, 186), the results are consistent with a single ordered pathway for the rearrangement of γ, then β, and then α genes in all T cells (Figure 10). In the proposed pathway, the V_γ genes rearrange and are transcribed first; around this time or soon after, rearrangement and transcription of joined D_β-J_β gene segments occurs,

followed by rearrangement and transcription of V_β gene segments, and then finally by rearrangement and transcription of V_α gene segments. If either D_α or D_γ gene segments exist, then further discrete steps may be identified.

The apparent regulation of gene rearrangement in T cells is consistent with the model described for immunoglobulin genes. As outlined above, according to this rearrangement model proceeds in a stepwise fashion; the result of a particular step is tested before proceeding to the next step. Applying this model to T cells, a productive V_β gene segment rearrangement may shut down further V_β gene segment rearrangement and activate V_α rearrangement through the synthesis of a β polypeptide. However, γ gene rearrangement may be regulated differently, because although this gene family rearranges early, data from helper cells suggests that productive γ gene rearrangement may not be required for further T-helper-cell development. We might therefore speculate that productive γ gene rearrangement is a developmental switch-point, in that cells with productive γ rearrangements tend to become either MHC class-I restricted or cytotoxic cells, while cells with only nonproductive rearrangements tend to become MHC class-II restricted or helper cells (Figure 10).

The Site of Rearrangement and Expression

INTRATHYMIC AND EXTRATHYMIC REARRANGEMENTS Several experiments strongly suggest that thymic precursors do not express T-cell antigen

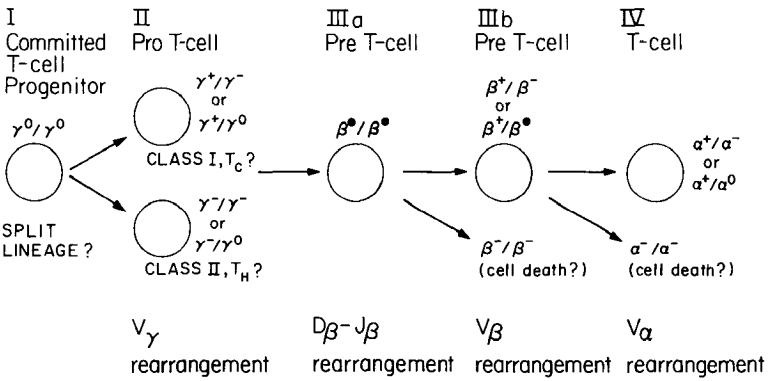

Figure 10 A schematic outline of four stages in the ontogeny of the rearrangement and expression of α, β, and γ genes. A zero superscript, as in "γ^0," represents a germline gene. A"+"superscript signifies productive rearrangement and a"−"superscript nonproductive rearrangement. The filled circle superscript represents a D-J rearrangement alone. The status of each homologue is illustrated; therefore, "γ^+/γ^-" represents a cell with one productive and one nonproductive γ gene rearrangement. The status of the gene rearranging at a particular stage is presented. Class I, T_C = MHC class I–restricted cytotoxic T cell. Class II, T_H = MHC class II–restricted helper T cell.

receptors and that α and β gene segment rearrangement and expression occur following migration to the thymus, although some γ gene rearrangement may occur prior to migration of precursors to the thymus.

Some fetal liver cells have γ gene segment but not β gene segment rearrangement. The discrepancy found between the lack of γ RNA in fetal liver and the observed rearrangements in these few fetal liver cells may be due to low levels of this transcript. The results of several experiments suggest that expression of α and β chains is initiated by gene rearrangement events that occur in the thymus. First, complete α and β transcripts cannot be detected in fetal liver, and they are not present in the most immature adult thymocytes or in fetal thymus until day 17 of gestation (146, 160, 173). Second, there appears to be a gradient of increasing β gene rearrangement in thymocytes as development progresses from day 14 through day 18 of gestation (146, 180). It is unlikely that these changes in the thymus DNA are caused by seeding of this organ by waves of increasingly mature precursors, because culture of day-14 and -15 thymus tissue has demonstrated that these cells can differentiate and attain immunocompetence in vitro (174, 175, 187–192). Although the possibility of some productive prethymic rearrangement of α and β genes cannot be formally ruled out, all the data are consistent with the hypothesis that thymic precursors do not express α and β polypeptides and that productive rearrangement and expression of these genes take place in the thymus.

NONPRODUCTIVE REARRANGEMENTS AND THYMIC T-CELL DEATH If the α and β genes rearrange in the thymus, and cells that have only nonproductive α or β gene rearrangements do not emigrate, then a large part of the intrathymic cell death that occurs in the adult thymus can be attributed to these nonproductive rearrangements. As mentioned above, two out of every three V_β gene segment rearrangements will be nonproductive because they would place the J_β gene segment in an inappropriate translational reading frame. This would mean that $0.67 \times 0.67 = 0.44$, or 44%, of the cells will have nonproductive β gene rearrangements on both homologues and therefore may not survive to emigrate from the thymus; the remaining 56% might go on to rearrange their α genes. If we assume either a direct V_α-J_α joining or a V_α-D_α-J_α mechanism similar to that observed in β genes, then two out of three V_α rearrangements will also be nonproductive. Again, 44% of the remaining T cells will have only nonproductive α gene rearrangements, and conversely, 56% of the remaining T cells will have a productive rearrangement. If we consider the progeny of a pre-T cell undergoing gene segment rearrangement in the thymus, no more than $0.56 \times 0.56 = 0.31$, or 31%, of the cells will produce a functional α-β heterodimer. Thus, a large percentage of the intrathymic cell death could be due to the lack of

successful rearrangements in the α and/or β genes. This hypothesis predicts that many of the cells that are destined to die in the thymus should not express T-cell antigen receptors on their surface. Recent experiments that have addressed this issue are not entirely conclusive. While it is certain that many of the cells with a surface phenotype thought to be characteristic of those that die in the thymus have α and β transcripts (185), there are conflicting estimates of the fraction of cells in this category that express α/β heterodimers (174, 193, 194).

GENERATION OF DIVERSITY IN THE VARIABLE-REGION GENES

The problem of generating diversity in the lymphocyte antigen receptor is common to both B and T cells. The immunoglobulin genes utilize three basic mechanisms: germline diversity, the utilization of a large number of different gene segments for variable-region gene formation; combinatorial joining, the random rearrangement of the different germline gene segments; and somatic mutational mechanisms that occur during and after the formation of the variable-region gene. A number of experiments have been conducted to determine to what extent these mechanisms are utilized in the T-cell antigen receptor.

Germline Diversity

BETA GENES The mouse germline β gene repertoire is composed of a relatively limited number of V_β and D_β gene segments and a relatively large number of J_β gene segments. Characterization of 37 expressed V_β sequences from murine thymus, spleen, T-cell hybridomas, and functional T-cell lines revealed only 16 different V_β gene segments (87–89, 148, 159). Because of the repeated use of V_β gene segments, and assuming that each gene segment is expressed with equal frequency, we calculated that the total number of functional germline V_β gene segments can be expected to be 24 or less, at the 95% confidence level (88; M. Meister, unpublished). Although the numerical calculation may be unreliable because the different V_β gene segments are not expressed with equal frequency (87), only a few V_β gene segments are expressed by many different clones in several different mouse strains; this would be unlikely if there were a large V_β gene segment repertoire. Southern blot analyses using the different V_β gene segment probes also support the notion of a limited repertoire (87–89). Surprisingly, several mouse strains have deleted a significant fraction of this repertoire and can still survive in the laboratory. In SJL mice, the best studied example, 10 V_β gene segments from 6 subfamilies have been deleted from the germline repertoire (R. Barth,

B. Kim, unpublished observations; D. Loh, personal communication; 89), and there is no evidence so far in favor of the theory that extra SJL V_β gene segments compensate for this deficiency (D. Loh, personal communication). However, studies on human β gene cDNA clones have indicated that the human V_β gene segment repertoire may be much larger than that of the mouse (P. Concannon, L. Pickering, P. Kung, L. Hood, unpublished).

Allowing for diversity arising from the recombination process, all of the D_β regions in murine V_β genes could be encoded by one of the two known germline D_β gene segments (87–89). However, in some cases only a few central nucleotides are shared with these D_β gene segments, and the existence of other germline D_β gene segments cannot be ruled out. Of the 14 murine J_β gene segments in the two J_β clusters, one in each cluster is a pseudogene and the other 12 are apparently functional (71, 77, 78). Of these 12 J_β gene segments, 10 have been found in the V_β regions that have been sequenced so far.

ALPHA GENES The germline V_α and J_α gene segment repertoires are larger than the corresponding β gene segment repertoires. A number of murine V_α gene segments obtained from cDNA libraries constructed from thymus and functional T-cell hybridomas have been sequenced (32, 33, 94, 95). The 25 different cDNA clones analyzed have 22 different V_α sequences that can be grouped into 11 subfamilies. Southern blot analyses with V_α probes from 9 different subfamilies reveal that there are at least 55 bands that cross-hybridize with these probes (32, 33, 94, 95; J. Klotz, unpublished), indicating that the germline V_α gene segment repertoire is larger than the V_β gene segment repertoire in mouse. The identification of 11 different V_α gene segment subfamilies is more than that observed so far in the murine immunoglobulin families and comparable to that of the β gene family. As noted previously, the J_α gene segment repertoire is much larger than the J gene segment repertoire in any of the other B- or T-cell receptor gene families (94, 95).

GAMMA GENES There are three V_γ gene segments that cross-hybridize with the V gene segment present in the original murine γ gene cDNA clone, and there are three murine J_γ gene segments (74). Although joining of these V and J gene segments permits nine different rearrangements, only three rearrangements account for most of those observed in murine T cells (146, 149). One of the rearranged γ gene restriction fragments that occurs frequently does not hybridize to the V_γ probe (149), and it therefore could involve another V_γ gene segment, or alternatively, it could result from a D_γ-J_γ rearrangement. The remaining two nongermline fragments hybridize with the V_γ probe (149). However, one of these rearrangements joins a V_γ to

a J_γ gene segment adjacent to the C_γ pseudogene (73) and accordingly is not functional. Therefore, it is possible that of the three predominant γ rearrangements, only one involving a particular V_γ and J_γ gene segment leads to the expression of a γ polypeptide. It is not clear why the other two known V_γ gene segments are rearranged only infrequently.

Combinatorial Joining

Analysis of expressed murine V_β genes and β gene rearrangements in T-cell hybrids and clones indicates that combinatorial joining occurs in β genes (87–89). For example, the $D_{\beta 1}$ gene segment joins to J gene segments in either cluster with approximately equal frequency (88, 180). In contrast, if we consider only rearrangement by deletion, the $D_{\beta 2}$ gene segment can only join to $J_{\beta 2}$ gene segments (87–89). The available sequences are also consistent with the hypothesis that V_β gene segments may join to any D_β-J_β rearrangements (87–89). If these V_β rearrangements are equally likely, there should be a bias in favor of $J_{\beta 2}$ and hence $C_{\beta 2}$ expression, because one germline D_β gene segment can join to either $J_{\beta 1}$ or $J_{\beta 2}$ gene segments, while the other can only join to $J_{\beta 2}$ gene segments. Such a bias in favor of $C_{\beta 2}$ expression is in fact seen in the analysis of different cDNA clones or of RNA from lymphoid tissues. There is also evidence supporting combinatorial joining of V_α and J_α gene segments (A. Winoto, unpublished). As noted above, the extent of combinatorial joining in the γ genes may be quite limited.

In addition to the random usage of gene segments, the β genes have an additional feature that may permit either optional or multiple usage of the D_β gene segments. The V_β gene segments have a two-turn recognition signal for DNA rearrangement (71, 72), the D_β gene segments have a one-turn recognition signal in the 5′ and a two-turn recognition signal in the 3′ flanking region (80–82), and the J_β gene segments have a one-turn recognition signal (71, 72, 77, 78) (Figure 3). In principle, the V_β gene segment can therefore rearrange directly to a J_β gene segment and still obey the one-turn to two-turn joining rule. Similarly, D_β-D_β joinings could perhaps create longer D_β regions. Either of these events would increase the diversity of the junctional region of the V_β gene. Analyses of different V-D-J junctions have identified a few potential V-J and D-D joining events (97, 98, 159), although there are other interpretations for these examples. However, if direct V_β-J_β joining or D_β-D_β rearrangements do occur, it is clear that these events are infrequent.

Somatic Mutational Mechanisms

The α, β, and γ genes employ three different mechanisms for somatic mutation that occur during the formation of the V gene. These mechanisms

also occur in the immunoglobulin V gene families, although the use of a fourth mechanism found in immunoglobulins is doubtful for the T-cell receptor.

JUNCTIONAL DIVERSITY The process that joins two gene segments together is imprecise. The joining event can delete nucleotides from the ends of the V, D, and J gene segments, leading to codon changes at the junctions of these segments (Figure 11). This process, called junctional diversity, occurs in both the immunoglobulin (123, 195, 196) and T-cell receptor (73, 75, 76, 81, 82, 148, 149) gene families.

N REGION DIVERSITY Additional nucleotides not encoded by either gene segment are added at the junction between the joined gene segments during rearrangement (Figure 11). This mechanism, known as N region diversification, is utilized by the immunoglobulin V_H genes, but not by the V_κ or V_λ genes (195, 197). N regions are also clearly present in the T-cell V_β genes (81, 82, 148); the occurrence of N region diversification in these four gene families is therefore correlated with the presence of D gene segments. However, this correlation may not include all six rearranging gene families because extra nucleotides are also seen in the V-J junctions of α and γ genes (73–76). It is not known whether these α and γ sequences arise from a combination of N region diversification and D gene segments, from D gene segments alone, or from N region diversification in the absence of D gene segments.

MULTIPLE TRANSLATIONAL READING FRAMES IN THE D GENE SEGMENT Analysis of the D_β regions of different V_β genes have shown that the two germline D_β gene segments are utilized with approximately equal fre-

	$V_{\beta 3}$	$D_{\beta 1}$	$J_{\beta 1\text{-}2}$
GERMLINE	CAGCAGTCTGTC	GGGACAGGGGGCC	CAAACTCC
3H.25	.....	TC.....	....
	$V_{\beta 3}$	$D_{\beta 2}$	$J_{\beta 2\text{-}5}$
GERMLINE	CAGCAGTCTGTC	GGGACTGGGGGGGCC	AACCAAGAC
2B4	.....	A.....A	.....

Figure 11 Junctional and N region diversity in functional V_β genes. Two rearrangements that include the $V_\beta 3$ gene segment, but different D_β and J_β gene segments, are illustrated; one from a lysozyme/I-A^b-specific T_H clone (3H.25), and one from a cytochrome c/I-E^k-specific T_H hybridoma (2B4) (71, 77, 78, 81, 82, 148). For each rearranged gene, the germline gene segments that comprise it are listed above, and the rearranged sequence below. For the rearranged sequence, nucleotides introduced by N-region diversification that are not in any of the germline gene segments are indicated, while nucleotides encoded in the germline are represented by a dot. A blank beneath a nucleotide encoded in the germline indicates those sequences deleted by the rearrangement event (junctional diversity).

quency in all three translational reading frames (88, 148) (Figure 12). Although this adds to the diversity of V_β gene segments, the low sequence complexity of the two germline D_β gene segments limits the contribution of this mechanism. In immunoglobulin V_H genes, however, the D_H gene segments are used in the same reading frame in most of the cases analyzed. The statistical preference for a single reading frame for the D_H gene segments cannot be accounted for simply by the presence of a stop codon in one reading frame of some of the D_H gene segments. The ability to use the D_β gene segments in three reading frames need not be considered an actual mechanism but may instead reflect the fact that there is little selection for particular protein sequences in this part of the molecule. Despite this coding sequence flexibility, the germline nucleotide sequences of mouse and human $D_{\beta 1}$ are identical; this suggests that there may be some selective pressure for the maintenance of a particular germline DNA sequence in this region of the β locus.

SOMATIC HYPERMUTATION Immunoglobulins employ an additional mechanism for diversification—somatic hypermutation—that generates base-pair substitutions throughout the V gene and its flanking sequences at a late stage in B-cell development (198–200). This mechanism is capable of

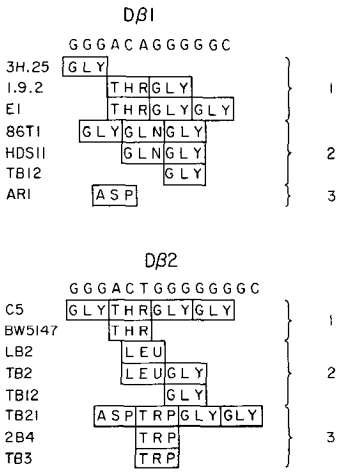

Figure 12 Ds in three. The D_β gene segments can be read in three reading frames. The nucleotide sequences of the two known D_β gene segments are shown along with the part of the D region amino acid sequence that could be encoded by germline D gene segments, as opposed to nucleotides due to N region diversification, from 15 V_β genes (31, 32, 34, 87, 88, 148). The reading frame for each set of translated sequences is shown on the right. Those sequences in the same reading frame are enclosed by a bracket on the right, and the reading frame is indicated by a number.

generating up to 3% sequence differences between the mutant and the corresponding germline gene segment (199). Those B cells with receptors that have higher affinities for antigen as a result of somatic hypermutation are believed to be selectively expanded when the antigen concentration is limiting (201).

Analyses of several different expressed V_β genes have shown that with the exception of the differences in the junctional regions, they are identical in sequence to their germline gene segment counterparts (71, 93, 148). Substantially more data is available if the nucleotide sequences of different cDNA clones are compared to one another, including several cases where no germline sequence is available. T cells expressing the same germline gene segments most often have identical gene segment sequences (71, 87–89, 159). In those cases where the sequences are not identical, there are very few differences in nucleotide sequence and never more than two substitutions at the amino acid level. The observed differences could be due to either somatic hypermutation or interstrain sequence polymorphisms, or they could be due to nucleotide sequencing errors (87–89). The preliminary nucleotide sequence data of V_α genes suggests that somatic hypermutation is also rare in this gene family. Two different sets of identical, expressed V_α gene segments have been found: the first from a collection of thymus cDNA clones (94) and the second from a series of cytochrome *c*/I-E^k-specific T hybridomas (A. Winoto, unpublished results). Because in each case either the three identical V_α gene segments were associated with different J_α gene segments or else the V_α-J_α junctional region was different, so the independent origin of each of these clones is established. Taken together, these data indicate that somatic hypermutation occurs infrequently, if at all, in the T-cell receptor genes.

There has, however, been a report of somatic variants arising in alloreactive T hybridomas in which new specificities were generated against class-II molecules after more than 200 cell generations in vitro (202). Because of the small number of mutant cells isolated, the frequency with which similar events occur has not been established. Therefore, it is not clear that the mechanism by which these mutants arise is comparable to that producing hypermutation in immunoglobulins, and the physiologic relevance of this provocative finding is uncertain.

Extent of T-Cell Receptor V, D, and J Diversity

Although the T-cell antigen receptor utilizes the same mechanisms for generating diversity as do the immunoglobulin genes, there are several differences in the extent to which these mechanisms are utilized. For example, the V_α and J_α gene segment repertoires are very diverse, but the murine T-cell receptor β gene family has a limited number of V_β and D_β

gene segments. Nonetheless, the total germline diversity potential in the V_β genes of normal mice may not be significantly less than that of immunoglobulin V_H and V_κ genes because of the relatively large number of J_β gene segments and the use of D gene segments in three different reading frames. Furthermore, many V_H as well as V_κ gene segments are very closely related and could be nearly equivalent functionally, while the few V_β gene segments are found in a relatively large number of diverse gene segment subfamilies. Some calculations of this diversity-potential for the murine T-cell antigen receptor and for immunoglobulins are presented in Table 3. If we assume that there are 250 V_H gene segments, 10 D_H gene segments, 4 J_H gene segments, as well as 250 V_κ gene segments and 4 functional J_κ gene segments (161), then a total of approximately 10^7 different antibodies could be synthesized. For the T-cell antigen receptor, if we assume that there are 30 V_β gene segments, 2 D_β gene segments used in all 3 translational reading frames, 12 J_β gene segments, 100 V_α gene segments, and 50 J_α gene segments, then a total of nearly 10^7 different T-cell antigen receptors are possible as well. Thus, using these reasonable estimates for the number of germline gene segments and assuming that the combinatorial mechanism for generating diversity can operate randomly, the magnitude of germline and combinatorial diversity possible for immunoglobulins and T-cell receptors is similar. Although the number of binding sites that can be generated is similar, clearly the composition of the gene segments involved is different. While the combined immunoglobulin κ and heavy chain families are estimated to have 500 V gene segments and only 8 J gene segments, the combined T-cell receptor α and β chains may have nearly as many J gene segments ($\sim$65) as V gene segments ($\sim$130). Because the 5′ ends of the J gene segments are diverse (Figure 6), T-cell receptors will have extra diversity just beyond the V-(D)-J junction, at positions homologous to those that fall within the third immunoglobulin hypervariable region. Thus, diversity is distributed differently in the variable regions of the calculated T- and B-cell repertoires, although the functional significance of this difference, if any, is unclear.

Perhaps one of the more surprising findings to emerge from the study of T-cell antigen receptors is that somatic hypermutation, a major mechanism for diversity generation in immunoglobulins, does not appear to play a significant role in the T-cell receptor genes. If this is true, then all the mechanisms for diversity generation in these V genes occur early in ontogeny during V gene formation in the thymus. There are three possible explanations for the failure to detect somatic hypermutation in the T-cell receptor genes. The first proposes that somatic hypermutation does occur but is not detected because the sequences analyzed were isolated mostly from cells that have not yet undergone somatic mutation (J. Howard,

Table 3 Potential diversity in murine T-cell receptor and immunoglobulin variable regions

	Immunoglobulins		T-cell receptor	
	H	L	β	α
Variable gene segment	250	250	30	100
Diversity gene segment	10	NA	2	—
D in three reading frames	NA[a]	NA	3	—
Joining gene segment	4	4	12	50
			$(30 \times 1 \times 3 \times 12)^+$	
Combinatorial joining	$250 \times 10 \times 4 = 1 \times 10^4$	$250 \times 4 = 1 \times 10^3$	$30 \times 1 \times 3 \times 6 = 1620$[b]	$100 \times 50 = 5000$
Combinatorial association	$1 \times 10^4 \times 1 \times 10^3 = 1 \times 10^7$		$1620 \times 5000 = 8.1 \times 10^6$	

[a] NA, not applicable; —, possible contribution, not yet established.
[b] The calculation assumes the $D_{\beta 1}$ rearranges to any of the 12 functional J_β gene segments, but that $D_{\beta 2}$ rearranges only to the 6 functional $J_{\beta 2}$ gene segments.

personal communication). Many of the sequences are derived from β chains synthesized by thymocytes that are relatively immature and therefore are perhaps not likely to have undergone somatic hypermutation. Moreover, many of the T-cell lines analyzed were stimulated in vivo with antigen only once and were then propagated in vitro under antigen-excess conditions. These would not, therefore, be subject to extensive selection for high-affinity receptor interactions associated with somatic hypermutation. A similar argument can be made for the T-cell hybridomas that do not require antigen to proliferate. Although the maturity of an antigen-stimulated B cell can to some extent be assessed by the immunoglobulin heavy chain isotype expressed (e.g. IgM vs others), there is no similar test for antigen-stimulated T cells. The second explanation proposes that somatic hypermutation in T-cell antigen receptor genes is not permitted because the resulting mutations, especially if they arise after T-cell migration from the thymus, could be deleterious to the organism (203). According to this view, since the T-cell antigen receptor repertoire has been selected for the recognition of antigen in the context of self-MHC-encoded molecules, T cells that undergo somatic hypermutation in the periphery will generate many autoreactive cells. As T cells on the average are believed to live longer than B cells, and T_H cells (unlike B cells) may not be dependent on other lymphocytes for activation and proliferation, these autoreactive T-cell somatic mutants present a greater threat than autoreactive B-cells. The third explanation proposes that somatic hypermutation events are rarely detected because of a lack of selection pressure for the expansion of the hypermutation events. This explanation implies that the activation of T cells is not highly dependent upon receptor affinity for antigen or an antigen/MHC complex (88).

MHC-RESTRICTED ANTIGEN RECOGNITION BY T CELLS

One or Two Receptors?

T cells recognize antigen when it is present on the surface of other cells only if the appropriate allele of a polymorphic MHC molecule is also expressed. Thus, T cells have a dual specificity for both antigen and allele-specific determinants of the MHC molecule (1–3), and the recognition of antigen is said to be restricted by the MHC-encoded molecule. This fundamental discovery was made more than a decade ago, and since then a debate has raged over the molecular basis of antigen and MHC recognition by T cells. Two types of models have been proposed to explain this dual specificity. The first hypothesizes that a single T-cell antigen receptor binding site

recognizes a combined determinant formed by the restricting MHC molecule and antigen (3, 204–206). This might occur if the antigen and the MHC molecule can interact with sufficient avidity to form a complex antigen. Recent experiments have shown that a peptide fragment of lysozyme capable of stimulating T_H cells can bind to an appropriate responder (but not the nonresponder) I-A molecule with significant affinity (207). The functional relevance of this binding to T-cell activation remains to be determined, and it is difficult to imagine that a similar, relatively high-affinity binding occurs for each of the antigens that are recognized in the context of a MHC class-I or class-II molecule. Alternatively, only a low affinity interaction between antigen and the MHC molecule may generally exist that is stabilized by the single binding site of the T-cell receptor (203). The second model proposes that the T-cell receptor is composed of either two separate molecules or two binding sites in the same molecular complex (208–213). In this case, separate binding sites exist for antigen and the MHC molecule.

Evidence has accumulated against the models in which antigen and MHC are recognized independently. First, independent segregation of antigen and MHC recognition was not found in somatic T-cell hybrids expressing two distinct antigen/MHC specificities (214). Second, when a panel of several hundred T-cell hybridomas was screened for reactivity with a clone-specific antibody that binds a particular α/β heterodimer, a second antibody-reactive clone was found that possessed the same antigen/MHC specificity, as well as a fortuitous allo-MHC cross-reactivity (215). This observation suggests that the same α/β heterodimer is involved in both antigen and MHC recognition. Finally, for both MHC class-I- and class-II-restricted T-cell clones, antigen recognition was shown to be directly influenced by the MHC haplotype expressed by the stimulating or target cells (216–219). For example, several instances have been described of T hybridomas that recognize peptides of cytochrome *c* and either one of two I-E molecules. The fine specificity of these individual T cells for a set of related cytochrome *c* peptides depended on which of the two possible I-E restriction elements was used to present antigen. Although for each of these experiments more than one interpretation has been proposed (220), these studies and others taken together imply that antigen and the MHC molecule are not recognized independently (214–219, 221–223), but that dual recognition is mediated through a receptor composed of α and β subunits. As we discuss below, the structure of the T-cell receptor itself argues against a model in which the α and β chains constitute two independent binding sites. This implies that a single receptor binding site can recognize both antigen and MHC.

Despite this evidence, the single-binding site model has not received

universal acceptance. The major unresolved difficulties with such models are the means by which MHC molecules can interact with a diversity of antigens to form a single determinant and the means of selection of a self-MHC-restricted repertoire of T-cell receptors. Although several hypotheses have been offered to account for these problems (204–206, 224, 225), none has won general acceptance. Despite problems with two binding site models, the discovery of the γ chain led to renewed speculation on the possibility of a second T-cell antigen receptor (212). The γ chain, although limited in diversity, could associate with the α or β chains (160, 212), or with a diverse, hypothetical δ chain, to generate a second T-cell antigen binding site. Alternatively, if the γ chain formed a homodimer or associated with a monomorphic molecule, it might act as an accessory molecule similar to Lyt-2.

Structural Comparisons Between Immunoglobulin and T-Cell Receptor V Genes

The V and C regions of the α, β, γ, and immunoglobulin polypeptides exhibit significant sequence similarity and have descended from common ancestral genes. Since the immunoglobulin molecule has been thoroughly analyzed at the level of primary, secondary, and tertiary structure (120, 226), it is pertinent to compare the structures of the T-cell receptor (α/β) and immunoglobulin V regions to gain insights into shared structural features relevant to binding of antigenic determinants.

SECONDARY STRUCTURE As noted above, immunoglobulin V regions fold into two planes of antiparallel β strands stabilized by the invariant disulfide bridge. The β strands are relatively conserved in sequence and are connected to one another by polypeptide loops, some of which constitute the hypervariable regions. The secondary structures of V_L and V_H regions are highly conserved. Several algorithms have been developed to assess the tendency of polypeptides of known sequence to have similar secondary structures. First, hydrophobicity analyses measure the hydrophobic characteristics of amino acid side chains (227). From this analysis, the exposure of side chains to the interior or exterior of the molecule can be inferred. Second, techniques have been devised for estimating the tendency for short regions of a polypeptide chain to form β-strand structures (228). Analyses of the average hydrophobicity or the β-strand forming potential of large collections of V_α, V_β, V_H, and V_κ regions have shown that all four sets of V region are similar to each other in these properties (87, 88, 94, 95).

AMINO ACID SIMILARITIES Invariant or nearly invariant amino acids are present at 8 positions in immunoglobulin variable regions, 5 in the V segment and 3 in the J joining region. At these positions, a single amino acid

is present in greater than 95% of both light (κ and λ) and heavy chain variable regions from several species (229). Four other positions are semi-invariant; a single amino acid is present in more than 75% of κ, λ, and heavy chain variable regions (229). In addition, there are a number of other positions where one set of variable regions, heavy or light, has a particular amino acid, and the other set does not (229). Fourteen conserved positions, 11 shared by both V_H and V_L regions, and three in which V_H and V_L regions are conserved within a family but differ from one another, are shown in Table 4. These 14 were selected from a larger group of conserved positions by two criteria: (*a*) The three-dimensional structures of immunoglobulins McPC603 and NEW indicate they might be important for V_H-V_L interactions (230, 231); and (*b*) the three-dimensional structure of both the NEW light and the NEW heavy chain indicates that these amino acids form hydrogen bonds that might be important for intrachain folding (231).

Many of the conserved amino acids in immunoglobulin variable regions are also present in the α, β, and γ chains. The invariant and semi-invariant amino acids in the β and α V gene segments are indicated above the blocks of sequence in Figures 4 and 5, respectively, and the invariant positions in the J_α, J_β, and J_γ gene segments are similarly denoted in Figure 6. Table 4 compares the V_α, V_β, and V_γ regions with immunoglobulins at the 14 conserved positions described above. Most of the conserved amino acids at these positions are also present in the three T-cell families of variable regions. For example, all of the 8 amino acids that are highly conserved (>95%) in both the V_H and V_L regions are also present in both V_α and V_β regions, and 7 are also present in the V_γ region. Of these highly conserved positions, 7 are indicated by asterisks in the left-hand column of Table 4, and the role these play in immunoglobulin intra-V-region folding and V_H-V_L interaction is indicated. The presence of these invariant amino acids, and most of the others listed in Table 4, in both V_α, V_β, and V_γ regions argues strongly that these variable regions are similar to immunoglobulins in overall structure.

INTERACTIONS BETWEEN V REGIONS The amino acids thought to be important for contact between immunoglobulin V_H and V_L regions include both variable and more conserved amino acids. Those contacts between two conserved amino acids on the light and heavy chain are likely to be most important for understanding the general features of V_H-V_L interactions. If we consider the three-dimensional structures of the mouse myeloma protein McPC603 and the human myeloma protein NEW, five amino acids on both the V_L and V_H regions are involved in such contacts (230, 231). Using the numbering of Kabat et al these include tyrosine-36L, glutamine-38L, proline-44L, tyrosine-87L, phenylalanine-98L in both κ

Table 4 Some conserved amino acids important for immunoglobulin variable region structure[a]

Number	Amino acid	Position in immuno-globulins	Presence in T-cell genes			Intrachain folding (H bond)	Interchain (V_H-V_L interaction)
			α	β	γ		
1*	CYS	23L, 22H	+	+	+	+	
2*	TRP	35L, 36H	+	+	+	+, with 12–13 positions C terminal	
3	TYR/VAL	TYR = 36L VAL = 37H	+ (TYR)	+ (TYR)	+ (TYR)	+, with TYR(PHE) 87L or 91H	+, with TRP 103H +, with PHE 98L
4*	GLN	38L, 39H	+	+	+	+, with amino acid 85L or 89H	+, with GLN 38L or 39H
5	PRO/LEU	PRO = 44L LEU = 45H	−	+ (LEU)	−		+, with TRP 103H +, with PHE 98L and TYR 87L
6	ASP	82L, 86H	+	−	+	+, hydrogen bonds to amino acid 79L or 83H	
7	ALA	84L, 88H	+	−	+	+, with VAL 109H or VAL, LEU 104L	
8*	TYR	86L, 90H	+	+	+	+, with THR 102L or 107H	
9	TYR(PHE)[4]	87L, 91H	−	−	+	+, with TYR 36L or VAL 37H	+, with PRO 44L or LEU 45H
10*	CYS	88L, 92H	+	+	+	+, with glycine 99L or 104H	
11	PHE/TRP	PHE = 98L TRP = 103H	+ (PHE)	+ (PHE)	+ (PHE)		+, with VAL 37H +, with TYR 36L
12*	GLY	99L, 104H	+	+	−	+, with CYS 88L or 92H	
13*	THR	102L, 107H	+	+	+	+, with TYR 86L or 90H	
14	VAL, LEU	104L, 109H	+	+	+	+, with ALA 84L or 88H	

[a] Fourteen conserved amino acids that may be important for intrachain folding and/or V_H-V_L interaction of variable region homology units. Immunoglobulin sequence data and numbering of the positions is according to Kabat et al (229). The first 10 positions are encoded by the V gene segment and the last four by the J gene segment. All conserved amino acids are present in >75% of both light and heavy chain sequences, except for positions 36L or 37H (≠3 in the left-hand column), 44L or 45H (≠5) and 98L or 103H (≠11), where V_L and V_H each have a different conserved amino acid, and at 104L, where light chains have either VAL or LEU. The asterisk in the left-hand column marks seven positions where the indicated amino acid is present in >95% of both all V_H and all V_L regions. Presence of the conserved amino acid in the V_γ gene from pHDS4, and in >75% of the V_α, J_α, V_β, and J_β gene segments, is indicated by a "+" under the appropriate column. If a conserved amino acid is involved in an intrachain hydrogen bond in both the light and heavy chains of the myeloma protein NEW, this is indicated by a "+" under the heading intrachain folding. If this H bond involves a second conserved amino acid on both chains, this is also indicated. Conserved amino acids that interact with other conserved amino acids to potentially stabilize V_H-V_L interactions are also indicated by a "+" under the appropriate column along with the amino acid involved in the interaction. Two amino acids are considered to be in contact if X-ray

and λ light chains, valine-37H, glutamine-39H, leucine-45H, tyrosine-91H, and tryptophan-103H in the heavy chain. Several of these amino acids are also highly conserved in α and β variable regions. The glutamine is found at the homologous position in both V_α and V_β regions. The V_β regions have leucine, and the V_α regions have either proline or leucine, at a position homologous to leucine 45H or proline 44L. On the other hand, the tyrosine (87L or 91H) is not commonly found in α and β variable regions, which often have a conservative substitution of phenylalanine or leucine at this position. At the two other conserved positions important for interchain contacts, both the V_α and V_β regions tend to have a tyrosine and a phenylalanine, and therefore resemble V_L regions somewhat more than they resemble V_H regions, which nearly always have valine and tryptophan at these positions (95) (Table 4). Furthermore, the distance between the cysteines that form the intrachain disulfide bond of the V_α and the V_β regions is 63–69 amino acids (95). Again, this property is more similar to the V_L regions, where this distance is 64–69 amino acids, than it is to V_H regions, where it is 69–75 amino acids (229). The α/β heterodimer may therefore resemble a light chain dimer more than it does a heavy-light chain pair. This is intriguing, because the structure of one light chain dimer that has been studied by X-ray analyses is unusual in that it has a very deep antigen-binding pocket (232). However, the tendency for V_α and V_β regions to be more similar to V_L regions is not uniform. In the length of their J gene segments and the presence of conserved amino acids homologous to alanine-92H and valine-111H, the V_α and the V_β regions are more similar to V_H than to V_L regions. It is therefore likely that the slightly greater resemblance of the V_α and the V_β regions to the V_L regions is due to convergent rather than divergent evolution of these V regions.

Antigen Binding by the α/β Heterodimer

HYPERVARIABLE REGIONS Calculations of variability, defined as the number of different amino acids occurring at a given position divided by the frequency of the most common amino acid at that position, permit the assessment of diversity at each position in a set of variable regions (233). An analysis of V_L and V_H regions with this approach reveals three distinct hypervariable segments—one around positions 24–35, a second near positions 50–65, and a third at the V-J or V-D-J junctions or positions 89–102 (233). The positions of the hypervariable regions correspond to those portions of the molecule that X-ray crystallographic studies have shown to be in contact with antigens (120, 226, 233). Examination of V_L and V_H region variability plots also reveals some additional regions of variability around positions 10–20 and 75–80, particularly in light chains, although these are not as variable as the three "classical" hypervariable

regions (229). The identification of hypervariable regions of T-cell receptor α and β chains is therefore expected to indicate those portions of the molecule involved in antigen/MHC contact. Such analyses have been carried out, and several general conclusions can be drawn. First, within the β chain, there is greater variability in positions homologous to those previously defined as hypervariable in immunoglobulins (87–89). Second, there is an overall higher level of background variability in both α and β chains, than in immunoglobulin light and heavy chains (87–89, 94, 95). Interestingly, hypervariability plots of collections of V_α regions have shown the variability to be broadly distributed throughout the variable region with the exception of the third hypervariable region, which is more variable, and a few scattered conserved positions (B. Arden, unpublished observation). This broader distribution of variability in both chains is consistent with other comparisons, indicating that there are fewer highly conserved amino acids in T-cell receptor V regions and that the average V_α and V_β regions are more different from one another than are immunoglobulin V regions (M. Kronenberg, unpublished observation; 87–89). However, the immunoglobulin sample used for comparison contains many myeloma proteins and hapten-binding proteins, and there is some evidence these may not represent the full extent of the diversity of the germline immunoglobulin repertoire (234–236). Third, it has been proposed that the V_β gene segments have several extra distinct hypervariable regions (87), although this was not observed in a second analysis (88). The discrepancy is best explained by differences in sequence alignments and the sensitivity of the analysis to slight sample differences when relatively small samples are employed.

Because of the apparent greater variability of V_α and V_β regions, and the possible existence of extra β chain hypervariable regions, it has been hypothesized that for the T-cell receptor a greater surface may interact with antigen than is the case for immunoglobulin (87). Although this is plausible, considering that the T-cell receptor is required to bind a complex determinant containing both antigen and MHC, there is too little structural information on the interaction of antibodies with protein antigens, as opposed to haptens, to definitively state that the two sets of receptors must be different from one another. The available crystallographic structure of an antibody-binding lysozyme suggests that a large surface may be involved in binding (237). This type of antibody-antigen interaction may account for the extra regions of immunoglobulin variability mentioned above and may well be comparable to the T-cell receptor interaction with antigen in the context of MHC.

THE α/β HETERODIMER : ONE OR TWO SITES? In summary, the V_α, V_β, V_H, and V_L regions all appear to exhibit a conserved set of amino acids that are

important in immunoglobulins for V_H-V_L interactions, a striking similarity in their patterns of secondary structure and similarity in their variability patterns. The data suggest that the antibody fold is conserved in the V_α and the V_β regions and that the V_α and V_β regions interact as in antibodies to generate a single binding site. In this regard, immunoglobulins apparently can be MHC restricted; several antibodies that recognize influenza polypeptides in the context of a specific MHC allele have been described (238). Accordingly, the antibody binding site has at least the potential for recognizing antigen in the context of an MHC molecule, and it is therefore possible that a single T-cell receptor could do the same. Alternatively, the α/β heterodimer may be responsible only for antigen or MHC recognition, in which case a second receptor needs to be defined.

Our view that the V_α and V_β regions fold in a manner similar to immunoglobulins to form a single binding site is not consistent with models that require the V_α and the V_β regions to form separate antigen and MHC binding sites, either as single chains (220) or in association with other molecules such as individual subunits of the Ia molecule (239). However, this single-site view of the α/β heterodimer can easily be reconciled with the lack of correlation between the expression of particular V_β gene segments and the ability to recognize either particular antigens or MHC molecules (Table 5). In one of the earliest studied cases, it was found that a T-cell hybridoma specific for cytochrome *c* and I-E^k, and a T-cell line specific for lysozyme and I-A^b, both express the $V_{\beta 3}$ gene segment (148) (Figure 11); however, other cytochrome *c*/I-E^k-specific hybridomas do not use this V_β gene segment (J. Goverman, A. Winoto, unpublished). Since then a number of similar cases have been described (Table 5). Because in immunoglobulins specificity can be associated with use of a particular light chain (240), a particular heavy chain (241), or both, we expect that the same will hold true for the T-cell receptor and that valid generalizations about α or β usage and antigen or MHC specificity will not be possible.

MHC Class I- and Class-II-Specific T Cells

There is a correlation between T-cell recognition of MHC class-I molecules, expression of γ gene RNA, and cytotoxic function on the one hand (149, 242–244), and a correlation between T-cell recognition of antigens in the context of MHC class-II molecules and helper function and/or IL-2 secretion on the other (242, 245–248). The reason for these correlations is unknown, although the structure of the T-cell antigen receptor in the two categories of cells is probably not responsible. Both T_H and T_C express C_α and C_β RNA, indicating that unlike immunoglobulins there are not function-associated T-cell-receptor constant-region isotypes. Furthermore, the T-cell receptors for both murine T_H and T_C cells appear to be encoded

Table 5 V_β genes expressed by functional murine T cells

T cell	Strain of origin	Specificity		V_β gene segments			Reference
		Antigen	MHC molecule	V_β	D_β	J_β	
1.9.2	B10.A(5R)	(Am-DAsp)	I-$E^{k/b}$	1	1	1.1	88
A20.2.15	(BALB/c × B6)F_1	Beef insulin	I-A^d	1	1		[2]
E1	BALB/c	trinitrophenyl	I-A^d	2	1	2.2	87
AR1	C57BL	*alloreactive*	H-2^{d1}	2	1	2.5	87
AOIT.8	B10.A	lysozyme (aa 74–86)	I-A^k	2	1	1.3	[3]
A10	B10.A	ovalbumin	I-A^k	2	1	1.2	86
2B4	B10.A	cytochrome *c*	I-E^k	3	2	2.5	71
3H.25	C57BL/6	lysozyme	I-A^b	3	1	1.2	148
V11.5	B10A(5R)	cytochrome *c*	I-$E^{k/b}$	3	1	1.2	[4]
LB2	C67BL/6	chicken RBC	I-A^b	6	2	2.3	87
AOIC.25.1	B10.A	lysozyme (aa 85–96)	I-E^k	6	1 or 2	2.2	[3]
3F9	BALB/c	alloreactive	D^b	6	1 or 2	1.1	159
2C	BALB.B	alloreactive	D^d	7	1	2.6	31
C5	C57BL/6	DNP-ovalbumin	I-A^b	8.1	2	2.5	87
AOIT.13.1	B10.A	lysozyme (aa 85–96)	I-E^k	8.3	1	1.1	[3]
V15.4	B10.A(5R)	cytochrome *c*	I-$E^{k/b}$	8.3	1	1.4	[4]
AOIC.9.4	B10.A	lysozyme (aa 85–96)	I-E^k	10	1	1.2	[3]
AOIC.19.3	B10.A	lysozyme (aa 85–96)	I-E^k	10	1 or 2	2.2	[3]
J6-19	B10.A	ovalbumin	I-A^k	14	1	2.3	86

[1] Specific for an undefined MHC class-I molecule.
[2] T. Wegmann, personal communication.
[3] J. Kobori, N. Shastri, unpublished data.
[4] J. Goverman, unpublished data.

by the same pool of V_β, D_β, and J_β gene segments. For example, analyses of a T-cell hybridoma specific for TNP and I-A^d and a cloned T-cell line specific for an H-2^d class-I molecule have shown that both utilize the $V_{\beta 2}$ gene segment in their antigen receptor (88) (Table 5). In addition, two monoclonal antibodies have been generated that each recognize a single V_β gene segment subfamily. KJ16-133 (52) is specific for the murine $V_{\beta 8}$ subfamily, which contains three members (87, 88), while Ti_{3A} (194) is specific for the human $V_{\beta M3}$ subfamily, which contains five members, three of which are functional (93). Both monoclonal antibodies bind both to L3T4$^+$ or T4$^+$ populations enriched for helper T cells and to Lyt-2$^+$ or T8$^+$ T-cell populations enriched for cytotoxic T cells. Therefore, cells of both phenotypes utilize the same V_β gene segments (179, 194). There is currently no data indicating whether the same might also be true for V_α gene segment usage.

Development of the T-Cell Specificity Repertoire

The ontological origin of a self-MHC restricted, antigen-specific repertoire of T-cell receptors has long been a controversial issue. It is now clear from experiments that determined the chromosomal locations of the α and β genes (Table 1) that these genes are not linked to those encoding the MHC molecules. It is therefore intriguing that MHC-congenic strains of mice with the same germline receptor genes express antigen-specific repertoires that in each strain are strongly biased for the recognition of different self-MHC molecules.

A number of studies, including those utilizing chimeric mice, have explored this question (249–251). Chimeric mice are constructed for this purpose by transferring precursor stem cells from the bone marrow of a mouse with one MHC genotype into another lethally irradiated mouse having a different MHC genotype. Stem cells in the bone marrow repopulate the hematopoietic system, and therefore in the chimera the hematopoietic cells have a different genotype than the irradiated recipient. Alternatively, chimeras have been constructed by transplanting thymus tissue into athymic mice that have a different MHC genotype. Many, but not all, of the chimera studies are consistent with a "thymic education" model for the ontogeny of a self-MHC restricted repertoire. According to this model, T cells with particular antigen-receptors are selected for in the thymus in the absence of antigen. This selection is believed to depend upon MHC molecules expressed by certain thymic cells; thus, T cells capable of recognizing antigens in the context of self-MHC molecules emigrate and populate the peripheral lymphoid organs. T cells that only recognize antigen plus allo-MHC, or that recognize antigen alone, do not emigrate and are presumed to die in the thymus. This selection process therefore

provides a rationale for the extensive cell death that takes place in thymus. According to several models of this type, selection is associated with a somatic hypermutation process (209, 210). The thymic education model contrasts with alternative antigen-priming models that assert that selection of T-cell specificities restricted by a given MHC haplotype occurs only in the periphery during T-cell activation in the presence of antigen (250). According to these models, mature cells emerging from the thymus are not particularly biased in favor of recognizing antigen plus self-MHC as opposed to antigen plus allo-MHC. However, in the peripheral lymphoid organs, the combination of antigen plus the available MHC molecules will lead to the clonal expansion of those T cells restricted to self-MHC molecules. Regulatory influences by suppressor and other cells may also prevent expansion of allo-MHC restricted clones.

Although study of the molecular biology of the α and β genes has not resolved this controversy, the available evidence permits the following conclusions. First, the thymus is the site where the α/β heterodimer is first expressed. Therefore, selection of receptor specificities for self-MHC recognition or induction of tolerance before the entry of T cells into the thymus is unlikely. Second, as mentioned above, up to 70% of the cell-death in the thymus may be caused by nonproductive α and β gene rearrangements. Some additional cell-death may also be due to the removal of self-reactive cells. However, these two explanations do not account quantitatively for the significant amount of cell death, estimated to be up to 95% of the total thymocytes. If the estimate is accurate, there must be additional causes for this phenomena (252–254). Finally, the absence of conclusive evidence for somatic hypermutation in both the α and β genes of the receptors expressed by peripheral T cells rules out models that require this as an obligatory step in the selection of a self-MHC-restricted T-cell repertoire. Therefore, the postulated selection of T-cell receptor specificities must act only upon the expressed receptor formed by germline genes and the diversity-generating rearrangement processes described above. However, none of these data argue directly for thymic selection as opposed to selection in peripheral lymphoid organs.

Implications for Immune Response Gene Defects

Immune response (Ir) gene defects are said to occur when heritable differences in responsiveness to the same antigen are demonstrated among individuals. In such cases, one group or individual are responders, while the other group manifesting the so-called "defect" are nonresponders (250, 255, 256). In most cases, it has been shown that responsiveness in inbred mice maps to MHC class-I molecules for cytotoxic cells (257) and to class-II molecules for helper T-cell responses (258). In these cases, nonresponsive-

ness is highly specific for the combination of particular antigens and particular alleles of MHC-encoded molecules.

Several models have been proposed to explain these Ir gene-encoded immune response gene defects (250, 255). One possibility is that germline genes that could encode a receptor with the specificity in question do not exist. A second category of models proposes that selective constraints lead to an absence of clones that recognize the combination of antigen-plus-MHC molecule. The requirement for self-tolerance is an example of such a selective constraint. Deletion of self-reactive clones could eliminate all those T cells that react with certain foreign antigen/self–MHC combinations, if the receptor expressed by these cells fortuitously cross-reacts with a self-determinant (259). A second explanation for this selective constraint proposes that selection of a self-MHC restricted repertoire in the absence of antigen (see above) leads incidentally to deletion of clones reactive with certain antigen/MHC combinations. The exact details of the relationship between repertoire selection and clonal deletion depend on whether one believes in one- or two-site models of the receptor (209, 210, 256). Explanations based upon a lack of germline genes or those requiring selective constraints both imply that T-cell responses that show Ir-gene control probably require the expression of relatively few genes, so that occasionally the gene(s) required for a particular response are not present or cannot be expressed.

A third hypothesis for Ir gene defects does not invoke an inability to express a particular T-cell specificity but proposes instead that certain antigen/MHC combinations may not effectively activate T cells in the periphery, although T cells that could react with these determinants are present. For example, the determinant selection theory states that a single T-cell receptor recognizes an antigen-MHC complex, but that in some cases a particular MHC molecule and antigen may fail to interact with the avidity required to form a complex and stimulate T lymphocytes (225). Another hypothesis invokes regulatory interactions between potentially responsive clones and suppressor cells that prevent responsiveness.

Two recent observations favor those explanations that do not require unavailability or deletion of clones expressing certain specificities, as is assumed by the prior two models. First, recent studies characterizing the specificity profiles of T-cell clones reactive to several small peptides have revealed considerable diversity among clones. For example, T-cell clones derived from B10.A mice immunized with a 23-amino-acid peptide of lysozyme (amino acids 74–96) showed a strict correlation between the minimal peptide determinants and the Ia molecule required for recognition; namely, clones specific for amino acids 74–86 were always I-A^k restricted, and clones reacting with amino acids 85–96 were I-E^k restricted

(260; N. Shastri, G. Gammon, A. Miller, E. Sercarz, unpublished). This correlation between the antigenic determinant and the MHC molecule also illustrates the phenomenon of Ir-gene defects, in that B10.A mice are nonresponders both to peptide 74–86/I-E^k and to peptide 85–96/I-A^k. Further analysis of the specificity profiles of these peptide-specific clones has shown that among each of the two sets restricted to different class-II molecules, individual clones can be distinguished by their reactivities to a set of variant synthetic peptides. Thus, it appears that T cells can recognize these small peptides along with the restricting MHC molecule, in several different ways.

The diversity of T-cell-specificity phenotypes observed for even minimal peptide determinants argues against absence of appropriate clones or clonal-deletion mechanisms as valid explanations for Ir-gene defects. For example, considering the self-tolerance model, given the diversity of the observed responses, it is difficult to see how fortuitous cross-reactions with a self-antigen could cause deletion of all the potential T cells reactive with the peptide. However, this could conceivably occur if the observed distinct specificity patterns of the clones were due to the fact that these clones had descended from a single parental clone through a somatic hypermutation mechanism. However, the sequencing of the V_β genes of the receptors employed by these clones has failed to reveal any somatic hypermutation, and different V_β sequences were found for each of the seven I-A^k- or I-E^k-restricted clones analyzed (J. Kobori, N. Shastri, unpublished). Similar diversity has been found for the β genes expressed in a series of cytochrome *c*–specific, T-cell hybrids (A. Winoto, N. Lan, D. Hansburg, unpublished data). This demonstrates that Ir-gene-regulated T-cell responses directed towards even minimal peptide determinants are not constrained in their usage of particular V_β genes, and it argues against the models described above, which predict that a relatively limited set of genes would be employed in these responses.

Another observation argues against the theory that the absence of clones with certain T-cell receptors is the cause of Ir gene defects. This is the finding that in one instance immune responsiveness to a different lysozyme peptide (amino acids 46–61) could be clearly correlated with binding of this peptide to an appropriate, but not to an inappropriate or nonresponder, MHC class-II molecule (207). This result is most consistent with the determinant selection hypothesis, although as mentioned previously, the generality of this finding has not yet been established.

In summary, these findings argue against a limitation in the existence or expression of appropriate T-cell receptor genes as a complete explanation for nonresponsiveness. To some extent, the studies cited support the

alternative explanations, including determinant selection and regulation by suppressor cells that assign the Ir defect to the activation of T cells in the periphery.

SUMMARY

The genes encoding the α and β chain of the T-cell receptor and the γ gene have been cloned, and their structure, organization, ontogeny of expression, pattern of rearrangement, and diversification are now generally understood. In most cases, the immunoglobulin paradigm applied very well to the corresponding phenomena in T cells, although as described above, some interesting and potentially important differences exist. Nevertheless, there are still many unanswered questions regarding the ontogeny and mechanism of MHC-restricted antigen recognition, and it is not clear how far the immunoglobulin model can take us in understanding these phenomena. Although the α/β heterodimer looks like an antibody and the binding sites of the two molecules may be similar, the rules governing B- and T-cell activation are clearly different, and the ligand(s) bound by the receptor are still poorly characterized. In the future, T-cell receptor genes, as well as those encoding the T-cell accessory molecules, will be altered in vitro and transferred into mammalian cells in culture and into whole organisms in an attempt to understand T-cell antigen recognition. These tools will allow us to manipulate the mammalian immune response in a variety of different ways that will have a profound impact both on our understanding of immunology and on medicine in the future.

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CONTENTS

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THE ROLE OF THE T3/ANTIGEN RECEPTOR COMPLEX IN T-CELL ACTIVATION

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INTRODUCTION

T lymphocytes can be activated to produce lymphokines, to express new cell-surface molecules, and, eventually, to proliferate. This activation of T lymphocytes is the result of ligand-receptor interactions occurring at the plasma membrane. As a consequence of these cell-surface interactions, signals are transmitted across the plasma membrane to the cellular interior. The T-cell antigen receptor plays a central role in these transmembrane signalling events. The triggering of the T-cell antigen receptor results in the generation of inositol trisphosphate (IP_3) and subsequent increases in the concentration of cytoplasmic free calcium $[Ca^{++}]_i$. Although the $[Ca^{2+}]_i$ signal alone is not sufficient to activate the cell, an increase in $[Ca^{2+}]_i$, together with other signals, sets in motion processes that result in new gene expression. This review will focus on the role of the T3/antigen receptor (T3/Ti) complex in human T-cell activation.

THE ANTIGEN RECEPTOR HETERODIMER

Of fundamental importance in understanding the events involved in T-cell activation is the identification and characterization of the cell-surface

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molecules involved. Antigen-induced stimulation and subsequent activation of the T cell must be initiated, in part, through interactions with the T-cell antigen receptor. The nature of this receptor has been elucidated through the use of monoclonal antibodies reactive with the receptor and the cloning of the genes encoding the receptor.

The T-cell antigen receptor was initially identified by the generation of monoclonal antibodies (MAb) that reacted with antigenic epitopes unique to individual human or murine T-cell clones, lines or hybridomas (1–6). These antibodies are thought to react with the antigen receptors of these cells since they are able to function either as agonists or antagonists for individual T-cell clones. For example, they can induce T-cell clone proliferation or lymphokine secretion (4, 6–8). Alternatively, they can block lymphokine production or cytolytic activity (2, 3, 5). Thus, such antibodies represent ligands interacting with clonally distributed antigenic epitopes involved in T-cell functions.

The antibodies react with disulfide-linked heterodimer glycoproteins of 80–90 kd (1–6). On human cells, the two chains consist of an acidic α-chain of 48–54 kd and a more basic β-chain of 40–44 kd (2, 6, 9, 10). The α- and β-chains are integral membrane proteins with peptide backbones of 30–35 kd with two and six possible N-linked glycosylation sites, respectively (11–13). There are only five intracytoplasmic amino acids for each chain. The antigenic polymorphism exhibited by the receptors isolated from different T-cell clones reflects the structural differences of α- or β-chains isolated from different cells. Peptide mapping studies of individual α- or β-chains isolated from different clones reveals that, although there are common peptide fragments observed in such digests, differences are also observed (10, 14, 15). These results suggest that the α- and β-chains contain both constant and variable regions. Indeed, such a motion has been confirmed by the characterization of their genetic structure.

A more complete understanding of the structure of the antigen receptor heterodimer (Ti) comes from the isolation of cDNA clones encoding these proteins. Subtractive hybridization was used to isolate cDNA clones for both the α- and β-chains (12, 13, 16–19). Such cDNA clones are unique to T cells, encode integral membrane proteins, and are rearranged when compared to nonlymphoid tissues in a manner analogous to immunoglobulin genes. The sequence of these cDNAs agrees with the sequencing data obtained with proteins isolated with monoclonal antibodies.

Clones encoding the β-chain were independently isolated by two laboratories (12, 16, 17). The β-chain exhibits considerable homology with the κ light chain of immunoglobulin, which suggests a common evolutionary origin. Genomic analysis reveals that, like immunoglobulin, the β-chain is assembled from distinct gene segments that undergo rearrange-

ment. Thus, variable (V), diversity (D), joining (J), and constant (C) gene segments have been identified (20–23). The human β-chain locus is encoded on chromosome 7 with two constant regions ($C_{\beta 1}$ and $C_{\beta 2}$), each with its own cluster of D and J gene segments (20, 22, 24). Two transcripts of 1.3 and 1.0 kb that are homologous to these cDNAs are detected by Northern blot analyses (12, 25). The 1.0 kb transcript is present in greater frequency in immature cells or tissues (26, 27). The 1.3 kb message represents a full-length transcript with leader, V, D, J, and C gene segments, whereas the 1.0 kb transcript does not contain a rearranged V gene segment (23, 28). The nature of the splicing sequences adjacent to the V, D, and J gene segments suggests that in the case of β-chain rearrangements, unlike that of immunoglobulin genes, direct joining of V and J gene segments might occur (20). Indeed, examples of cells containing direct V-to-J joining have been reported (28). Such an alternative system of joining would allow for increased diversity.

The α-chain was isolated by subtractive hybridization and partial amino acid sequencing in the mouse and human (13, 18, 19, 29). Homology to both the β-chain and immunoglobulin genes suggests that all these proteins belong to a supergene family (30). Variable and constant gene segments appear to undergo rearrangements in a manner analogous to the β-chain (18, 19). The genomic analysis of the α-chain gene has not been completed. The α-chain is present on the *fourteenth* chromosome in mouse and man and is tightly linked to the purine nucleoside phosphorylase gene, a deficiency of which is associated with immunodeficienicies in man (31, 32). Moreover, translocations of the fourteenth chromosome are commonly seen in some T-cell malignancies, raising the question of the role of sequences in or near the α-chain gene in the transforming event. The α-chain transcripts are 1.5 and 1.3 kb in length, and the 1.3 kb transcript appears to lack V gene segments (13). Studies in the mouse demonstrate that the α-chain transcripts appear later during thymocyte ontogeny than do β-chain transcripts (26). These observations are of particular interest in light of the studies discussed below, which suggest that the β-chain may regulate the level of α-chain transcripts.

Another cDNA, the γ-chain, has been isolated; it is T-cell specific, undergoes limited rearrangements, and has considerable homology to the β-chain (33). Its role as an antigen receptor molecule, however, is not clear. Transcripts encoding this gene are more frequently detected in cytolytic T lymphocytes than in other T-cell subpopulations and appear to be more abundant early in T-cell ontogeny (26, 34). Unlike the α- and β-chain genes, there are no N-linked glycosylation sites (33). The γ-chain has limited variability and has not been detected on the surface of cells (34). The failure to detect this molecule may be the result of the lack of monoclonal

antibodies reactive with it. Alternatively, given its restricted distribution and its limited variability, it may have a different, as yet unidentified, function confined to the cell interior.

THE STRUCTURE OF T3

Associated with Ti on human cells are the three T3 peptides that have been termed T3-γ (25 kd), T3-δ (20 kd glycoprotein), and T3-ε (20 kd protein), according to their apparent molecular weights (35, 36). The 25-kd T3-γ has been found in immunoprecipitates with all anti-T3 reagents. This glycoprotein, which has a 16-kd polypeptide chain backbone, appears structurally different from the other T3 proteins (37, 38). T3-γ is susceptible to Endoglycosidae(Endo)-F, but not to Endo-H, and is a transmembrane protein (38–40). Whether this is a T cell–specific component is unknown. As T3-γ is sometimes weakly represented in immunoprecipitates prepared with anti-T3 reagents, it is assumed that none of the known monoclonal reagents react with the T3-γ chain.

Several experiments indicated that two 20-kd T3 proteins exist (T3-δ and T3-ε). Two hydrophobic reagents, ^{125}I-iodo-naphthyl-azide (^{125}INA) and the carbene 3-(trifluoromethyl)-3-(m-[^{125}I]-iodophenyl) diazirine (^{125}I-TID), label the T3-ε preferentially (39; R. Malin, C. Terhorst, unpublished). In fact, no counts were detected in the α, β, or γ-chains, whether the labeling reagent was offered to intact cells or to isolated plasma membranes. The largest part of the 20-kd T3 material that could be labeled with ^{125}I-TID or ^{125}INA was not susceptible to Endo-F. Analysis by isoelectric focusing showed that most of ^{125}I-TID material was found in one band. About 10% of the counts were present in the area of the T3-δ chains. These experiments strongly support the notion that one of the two 20-kd T3 chains is a nonglycosylated membrane protein, which can be labeled preferentially by hydrophobic reagents. Further evidence for the existence of a nonglycosylated 20-kd T3 species was derived from pulse/chase kinetic experiments, peptide maps, and N-terminal sequence analysis (36, 39).

To obtain another means of positive identification of the T3-ε chain, monoclonal antibodies were prepared by immunizing mice with purified denatured 20-kd T3. Two of these antibodies (SP6 and SP10) detected a 20-kd T3 (T3-ε) in an immunoblotting experiment after treatment with Endo-F (41). In order to establish that hydrolysis with Endo-F was complete, a control experiment was included using a rabbit antiserum (6081) that had been raised against a synthetic peptide with the amino acid sequence 1–14 of the 14-kd T3. Conversely, five monoclonal antibodies recognized the 14-kd T3 chain. To test the tissue specificity of the anti-T3-δ and anti-T3-ε monoclonal reagents, frozen tissue sections of human thymus, lymph

nodes, and tonsils were stained using a sensitive indirect immunoperoxidase technique. Results from these experiments strongly suggest that both T3-δ and T3-ε are T-cell specific. T3-ε is present in all thymocytes, whereas T3-δ is expressed more strongly on medullary cells than on cortical cells.

Recently, cDNA clones coding for the T3-δ chain were isolated (42). Several conclusions can be drawn from the amino acid sequence that was derived from the nucleotide sequence of these cDNAs. The T3-δ protein consists of three domains, an extracellular domain (residue 1–79), a transmembrane segment (80–106), and an intracellular domain (107–150). The transmembrane segment consists of a stretch of 27 predominantly hydrophobic amino acids (residues (80–106) that is interrupted by an aspartic acid (residue 90). Asp 90 could form a salt bridge with the Lys residue in the transmembrane segment of the T3/T-cell receptor α- or β-chain (12, 16–19). This type of bond would be pronounced, due to the low dielectric constant of the hydrophobic environment. The extracellular domain contains two N-linked oligosaccharides. It is unlikely that the T3-δ chain has a hairpin configuration, because protease K experiments with "inside-out microsomal vesicles" showed that all components present in anti-T3 immunoprecipitates span the plasma membrane (40). The cytoplasmic domain is large in relation to that in the extracellular compartment, when compared with the T-cell receptor α- and β-chains. This may reflect the signal transferring function of the invariable T3 chains as opposed to the ligand binding function of the clonotypic T-cell receptor heterodimer.

The primary translation product of the T3-δ chain is 19 kd, including a 2200-M_r signal peptide. Combined with our previous finding that deglycosylation results in a 16-kd and a 14-kd product, this observation strongly suggests a proteolytic processing step (36, 38). Since the 14-kd and 16-kd T3 have the same N-terminal sequences, the proteolytic processing must occur in the C-terminal cytoplasmic portion of the T3-δ chain (36, 42). That this proteolytic processing plays a role during the activation of T lymphocytes following the binding of antigen to the T-cell receptor is an appealing speculation in view of the role of T3 in T-cell activation.

Southern blotting experiments using several restriction enzymes showed that the T3-δ gene is a single-copy gene (42). The T3-δ gene has been mapped to human chromosome 11 by hybridization of a T3-δ cDNA clone to DNA from a panel of human/rodent somatic cell hybrids (43). In contrast, the gene for the human T-cell receptor β-chain was found on chromosome 7 (24). In Southern blotting experiments with DNAs of somatic cell hybrids that contained segments of chromosome 11, we have been able to assign the T3-δ gene to the distal portion of the long arm of chromosome 11 (11q23-11qter) (43).

Murine T cells also contain at least the T3-δ chain, a fact confirmed by a

cDNA clone coding for the murine T3-δ chain that was recently described (44). Several conclusions can be drawn from the comparison of amino acid sequences of human and murine T3-δ. A high level of amino acid sequence conservation occurred in both the transmembrane (70.4%) and intracellular (79%) domains of murine and human T3-δ. Much less was found in the extracellular domain (57.5%).

The relative position of the cysteine residues and of two attachment sites for Asn-linked glycosylation are conserved in the extracellular domain of the molecule. The murine T3-δ chain contains a third consensus sequence (Asn^{34}-Lys^{35}-Thr^{36}) for N-linked glycosylation. This would predict that the mature glycosylated murine T3-δ chain has a higher molecular weight than its human equivalent. The amino acid sequences around Asn-34 are very different from those in the human polypeptide chain. This suggests that an antigenic determinant, recognized by murine monoclonal antibodies, could be located in the human polypeptide chain between residues 20 and 39. Since this area of the molecule carries an oligosaccharide in the murine chain, this putative determinant could be located on the surface of both the human and the murine protein. The existence of an easily accessible determinant on the surface of the human molecule would explain why anti-T3 monoclonal antibodies have been prepared with a relatively high frequency. Another potential site for an antigenic determinant on the human T3-δ chain is around amino acid 60.

The assignment of murine T3-δ to mouse chromosome 9 is not surprising, since the human T3-δ gene had been mapped to the long arm of human chromosome 11 (11q23-11qter). Linkage studies in mice have indicated that at least a 4cM-long part of the long arm of human chromosome 11 is homologous to mouse chromosome 9 (45). Interestingly, in the mouse another T cell–surface antigen, Thy-1, is linked to UPS, esterase 17, and ALP-1, in the order: esterase 17/UPS/ALP-1/Thy-1. The synteny of human chromosome 11 and mouse chromosome 9 would predict that the human Thy-1 gene is located on the long arm of chromosome 11. Conversely, the mouse T3-δ chain gene would map to the UPS-Thy-1 region of chromosome 9.

ASSOCIATION OF T3 AND THE ANTIGEN RECEPTOR HETERODIMER AS A MULTI-SUBUNIT COMPLEX

Several lines of evidence support the notion that the T-cell antigen receptor exists as a molecular complex composed of the α/β-chain heterodimer and T3 on the plasma membrane: (*a*) Ti comodulates with T3 antigens on T-cell clones and lines (2); (*b*) antigen-induced unresponsiveness of T-cell clones is

associated with a diminished expression of T3 (46); (*c*) the appearance of T3 and Ti is linked during T-cell ontogeny (15); (*d*) under some circumstances, immunoprecipitation of T3 results in coprecipitation of Ti (9); and (*e*) T3 and Ti have been chemically cross-linked using bifunctional reagents (47, 48). The exact structural and functional relationships between Ti and T3 have not been defined. Cross-linking studies suggest a structural association between the β-chain of Ti and the γ chain of T3 (47). In these studies, cleavable and noncleavable reagents cross-linked the T3-γ-chain to the β-chain. However, the three chains of T3 were not cross-linked, nor were the α- and β-chains of Ti. Therefore, the ability of these reagents to cross-link the T3-γ-chain and the β-chain may simply reflect a spatial relationship between two free amino groups, rather than an actual molecular association between these two chains. Therefore, the structural relationship between Ti and T3, as well as their functional interactions, is not clear.

In an effort to study the structural and functional relationships between T3 and Ti, we attempted to derive mutants of the T3-bearing leukemic line, Jurkat, which lacked either T3 or Ti (6). To accomplish this, Jurkat cells were mutagenized either with ethyl methane sulfonate (EMS) or gamma radiation. Cells were negatively selected with either anti-T3 or anti-Ti (C305) plus complement. Cells that failed to express the cell-surface antigens selected against were isolated using indirect immunofluorescence and a fluorescence-activated cell sorter. Cells were shown to be negative for the relevant cell-surface antigen by indirect immunofluorescence, antibody- and complement-mediated cytolysis, and quantitative absorption. In four separate mutagenization experiments in which more than 50 clones were analyzed, the identical phenotype was found regardless of the antibody used in the selection procedure. All cells, whether they were selected for T3 negativity or Ti negativity, fail to express both T3 and Ti (Figure 1, from 6). Moreover, the failure to detect Ti is not due to an alteration in the conformation of the antigen receptor heterodimer. The disulfide-linked heterodimer representing Ti can not be detected on diagonal gels of whole-cell lysates containing iodinated cell-surface proteins of all of the mutants analyzed. These studies suggest an oligate requirement for the coexpression of T3 and Ti proteins on the cell surface.

The underlying defect that results in the failure of these mutants to express either Ti or T3 was explored in an attempt to understand the relationship between T3 and Ti (49). Biosynthetic labeling studies reveal that all mutants analyzed contain intracellular T3 proteins that can not be distinguished from proteins isolated from the wild-type cell (49). Nonetheless, these cells fail to express these T3 proteins on their plasma membranes. These results suggest that another defect in these cells prevents the expression of T3 on the cell surface and may involve the antigen

receptor heterodimer. Indeed, Northern blot analysis reveals that three of seven independently derived mutants fail to contain the 1.3 kb full-length β-chain transcript of the antigen receptor (49). They all contain the 1.0 kb transcript also detectable in the wild-type Jurkat cell. These same three mutants also contain a diminished level of α-chain transcripts; this suggests that the β-chain regulates the level of α-chain transcripts (49). The presence of T3 proteins detected intracellularly in mutants lacking the full-length β-chain transcript suggests that the expression of an α/β-chain heterodimer is a requirement for the cell-surface expression of T3 glycoproteins. This supports an intimate structural relationship between T3 and Ti. A prediction of such a hypothesis is that reconstitution of the β-chain of Ti should result in cell-surface expression of T3 as well as the heterodimer.

To establish that cell-surface expression of T3 requires the expression of

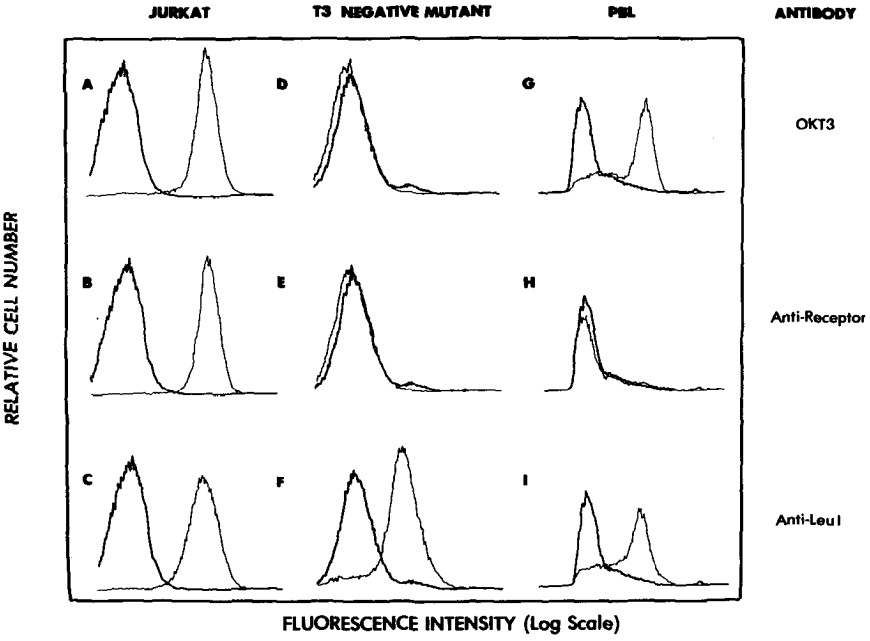

Figure 1 A mutant of Jurkat selected for T3 negativity also fails to express antigen receptor determinants. Shown are flow cytometer fluorescence histograms of Jurkat, S.5 (a mutant selected for T3 negativity), and peripheral-blood lymphocytes stained by indirect immunofluorescence with the indicated antibodies. The heavy lines in each panel are the control histograms obtained with a nonreactive monoclonal antibody, and the light lines are the histograms obtained with the indicated antibodies.

Ti, we attempt to reconstitute one of the mutants that lacks the full-length 1.3 kb β-chain transcript (49). YT35, a cDNA of the full-length β-chain gene isolated from Molt 3 was found to be identical in sequence to the β-chain gene isolated from Jurkat (28). A plasmid was constructed that contained YT35 under the influence of the Friend spleen focus forming virus, long terminal repeat and the neomycin resistance gene (49). By protoplast fusion, this plasmid was introduced into J.RT3-T3.5, a radiation-induced mutant of Jurkat that lacks the 1.3 kb β-chain transcript and has a diminished level of α-chain mRNA (49). Of 15 clones resistant to geneticin, 7 express abundant levels of antigenic determinants detected by the anti-Ti monoclonal antibody, C305. Moreover, all these cells also reexpress T3 antigenic determinants. Southern blot analysis reveals that such transfectants contain new sequences homologous with the β-chain, not seen in either Jurkat or the recipient mutant cell. Northern blot analysis confirms that such cells contain new transcripts homologous to the β-chain ot YT35 but different in size from the 1.3- and 1.0-kb transcript seen in Jurkat. These results demonstrate that insertion of a β-chain gene, which results in reconstitution of Ti, also results in reexpression of T3 glycoproteins on the cell surface. Thus, these results establish that cell-surface expression of T3 depends on cell-surface expression of an antigen receptor heterodimer.

Collectively, these observations support the model of the T-cell antigen receptor as a multisubunit structure consisting of T3 and Ti (Figure 2). Whereas, the function of antigen receptor heterodimer must certainly involve an interaction with antigen, the role of T3 in this multimeric complex is not clear. Whether it contributes to antigen binding, correct conformation of the receptor, or transduction of the activation signal remains to be explored.

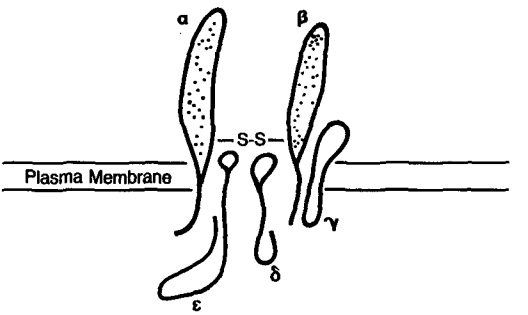

Figure 2 The T-cell receptor/T3 complex. Model of the T3/Ti complex as a multimeric structure.

THE ROLE OF THE T3/ANTIGEN RECEPTOR COMPLEX IN T-CELL ACTIVATION

In vivo, T cells are activated by antigen presented by macrophages in the context of the major histocompatibility antigens. Such an interaction must involve the T3/Ti complex. In vitro, however, T cells may be activated not only by antigen on appropriate antigen-presenting cells, but also by other ligands interacting with the T3/Ti complex. Thus, monoclonal antibodies directed against either T3 or Ti can activate T cells (50, 51). Under appropriate circumstances, they can induce proliferative responses and the secretion of lymphokines (50–53).

In addition to antigen or antibodies reactive with T3 or Ti, lectins such as PHA or Con A have been used to activate T cells. We examined the ability of PHA to activate the T3/Ti negative mutants of Jurkat described above (6, 54). Like peripheral T cells, Jurkat requires two stimuli for activation (53). For both peripheral T cells and Jurkat, one stimulus involves a ligand interaction involving a T3 or Ti. In the case of peripheral T cells, the second stimulus appears to be provided by macrophages and may be interleukin-1 (IL-1) (55–57). Jurkat and peripheral T cells can also receive this second stimulus by the addition of phorbol myristate acetate (PMA) (53, 54, 58, 59). Thus, anti-T3 or anti-Ti induce Jurkat to produce IL-2 if added together with PMA. Similarly, if Jurkat is stimulated with the combination of PHA and PMA, it will produce abundant levels of IL-2 (53, 54). In contrast, mutants of Jurkat that do not express the T3/Ti complex fail to produce IL-2 in response to PHA and PMA (6, 54). These results suggest that cell-surface expression of the T3/Ti complex is required for PHA-induced activation of Jurkat. An alternative explanation is that these cells lack the ability to produce IL-2 due to other intrinsic defects. Therefore, it is necessary to show that such cells have the ability to produce IL-2.

Since calcium ionophores are mitogenic for peripheral T cells, we attempted to bypass the requirement for interactions involving cell-surface molecules by utilizing calcium ionophores (58). Jurkat can be activated to produce IL-2 in response to two different calcium ionophores (A23187 and ionomycin), but only if PMA is also added (6, 54, 59). Moreover, we were able to activate, with a Ca^{2+} ionophore and PMA (6, 54), the Jurkat mutants that fail to express the T3/Ti complex. These results suggest that responsiveness to PHA requires the cell-surface expression of the T3/Ti complex. More direct evidence for this notion is provided by the observation that the transfected cell, PF-2.8, which expresses a re-constituted T3/Ti complex also now responds to the combination of PHA and PMA (Table 1). These data provide strong evidence to suggest that the mitogenic action of PHA involves a direct or indirect interaction with the

Table 1 Reconstitution of PHA responsiveness in the transfected cell, PF-2.8[a]

Cell	Supernatant dilution	IL-2 production in response to PMA	PHA + PMA CPM	PHA + PMA A23187 + PMA
Jurkat	1:4	1,742	58,422	59,490
	1:8	622	54,674	55,778
	1:16	248	55,004	55,682
J.RT3-T3.5	1:4	857	2,294	50,768
	1:8	284	1,248	48,338
	1:16	188	140	32,644
PF-2.8	1:4	1,978	53,222	48,228
	1:8	1,644	33,964	34,922
	1:16	244	20,008	19,732

[a] Jurkat, J.RT3-T3.5, or PF-2.8, 10^6 cells/ml, were stimulated with PMA (50 ng/ml) in the presence or absence of PHA (1 μg/ml) or the calcium ionophore A23187 (1 μg/ml) for 24 hr. Culture supernatants were collected and serial two-fold dilutions were prepared. To each dilution, 3000 IL-2 dependent cells, CTLL-20, were added and cultured for 24 hr. One μCi of ^{3}H-thymidine was added during the last 4 hr of culture, and the -thymidine uptake determined.

T3/antigen receptor complex. Supporting this notion is the recent finding that Ti proteins are included among the proteins to which PHA binds on human T cells (60).

PERTURBATION OF T3/Ti INCREASES $[Ca^{2+}]_i$

An increase in the concentration of cytoplasmic free calcium $[Ca^{2+}]_i$ can function as the "second messenger" by which receptor-ligand interactions regulate cellular activities (61). Ca^{2+} ionophores, which increase $[Ca^{2+}]_i$, can substitute for lectins or MAbs to T3/Ti in activating Jurkat and can bypass the requirement for the expression of T3/Ti on the mutants (6, 54, 59). These observations suggest that increases in $[Ca^{2+}]_i$ play a role in antigen receptor–mediated activation.

Because MAbs to the T3/Ti complex on Jurkat act as agonists, we used these MAbs to study the mechanism by which the antigen receptor transmits a signal or signals across the plasma membrane in order to initiate activation (6, 54, 59, 62). This approach makes it possible to study directly the signalling capabilities of T3/Ti complex itself and avoids the ambiguities inherent in using nonspecific stimuli such as lectins.

To determine whether perturbation of T3/Ti increases $[Ca^{2+}]_i$, we monitored $[Ca^{2+}]_i$ with the Ca^{2+}-sensitive fluor, quin2. In unstimulated quin2-loaded Jurkat cells, $[Ca^{2+}]_i$ is approximately 80 nM (54, 59, 62). The

addition of MAbs to either T3 or to Ti results in a prompt, sustained increase in $[Ca^{2+}]_i$ (Figure 3) (54, 59, 62). Under optimal conditions, $[Ca^{2+}]_i$ increases 20 sec after the addition of the MAb to the cellular suspension, reaches a peak of 750 nM within 45 sec, and then falls to a "plateau" that remains elevated above basal levels for at least 1 hr (59). Although PMA lowers $[Ca^{2+}]_i$ somewhat, T3/Ti MAbs induce substantial increases in $[Ca^{2+}]_i$ in PMA-treated Jurkat cells (59). In marked contrast to the effects of the Ti MAbs, MAbs to other cell-surface determinants (Leu 1, Leu 5, T4, HLA class I) on Jurkat do not affect $[Ca^{2+}]_i$ (54, 59). Concentrations of the Ca^{2+} ionophore, ionomycin, that synergize with PMA in activation lead to increases in $[Ca^{2+}]_i$ in quin2-loaded Jurkat cells that are comparable to those induced by perturbation of T3/Ti (59). The antigen receptor–mediated increase in $[Ca^{2+}]_i$, then, appears to function as a physiological signal for activation in Jurkat cells.

Antigen receptor–mediated increases in $[Ca^{2+}]_i$ are not limited to Jurkat but can be demonstrated in other T-cell malignant lines, as well as in T-cell clones (62–65). In addition, perturbation of T3 can increase $[Ca^{2+}]_i$ in polyclonal T-cell populations. For example, 64.1, a MAb to T3, induces a two-fold increase in $[Ca^{2+}]_i$ in bulk cultures of human peripheral-blood T cells (J. Imboden, J. Stobo, unpublished). Similarly, O'Flynn et al have found that MAbs increase $[Ca^{2+}]_i$ in freshly isolated T cells (63). This important observation suggests that perturbation of T3/Ti increases $[Ca^{2+}]_i$ in normal, resting T lymphocytes.

PHA increases $[Ca^{2+}]_i$ in T lymphocytes (66). Similarly, in Jurkat cells, the addition of this lectin results in substantial, sustained increases in $[Ca^{2+}]_i$ (54). PHA does not affect $[Ca^{2+}]_i$ in mutants that fail to express T3/Ti (54). Therefore, the ability of PHA to increase $[Ca^{2+}]_i$ in Jurkat cells,

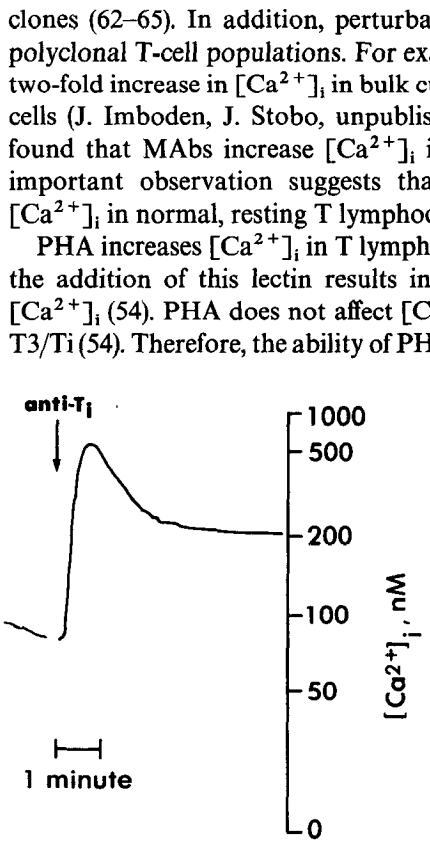

Figure 3 A monoclonal antibody to T increases $(Ca^{2+})_i$. Jurkat cells were loaded with the Ca^{2+}-sensitive indicator, quin-2 and the fluorescence of the cellular suspension was monitored over time. C305, a monoclonal antibody to Ti, was added at the indicated time. $(Ca^{2+})_i$ was calculated by the method of Tsien et al (8).

as well as to activate Jurkat, requires the cell-surface expression of T3/Ti. The density of the E-rosette receptor that is expressed by these mutants is identical to that of the wild-type Jurkat (6). This is noteworthy as MAbs to the $T11_3$ epitope of the E-rosette recepetor can increase $[Ca^{2+}]_i$ (64). Although PHA may interact with the E-rosette receptor on peripheral T cells, it is quite clear that, with Jurkat, interaction between the E-rosette receptor and PHA is not sufficient to $[Ca^{2+}]_i$ (6, 54, 67).

THE MECHANISM OF THE T3/Ti-MEDIATED INCREASE IN $[Ca^{2+}]_i$

Because the T3/Ti-mediated increase in $[Ca^{2+}]_i$ appears to function as a physiologically significant signal for T-cell activation, the mechanism by which the antigen receptor complex increases $[Ca^{2+}]_i$ becomes one of critical importance. Receptor-mediated increases in $[Ca^{2+}]_i$ can be due to the influx of extracellular Ca^{2+}, to release of Ca^{2+} from intracellular stores, or to a combination of these mechanisms. To determine whether perturbation of T3/Ti mobilizes intracellular Ca^{2+}, we tested the ability of the T3/Ti MAbs to increase $[Ca^{2+}]_i$ when extracellular Ca^{2+} is depleted (62). The addition of 10 mM EGTA to a suspension of quin2-loaded Jurkat cells reduces the concentration of extracellular Ca^{2+} from its normal level of 1 mM to less than 60 nM (62). Under these conditions, the addition of T3/Ti MAbs to quin2-loaded Jurkat cells increases $[Ca^{2+}]_i$ to a peak level that is comparable to that seen in the presence of 1 mM extracellular Ca^{2+} (62). This increase in $[Ca^{2+}]_i$, occurring in the absence of the Ca^{2+} gradient of 10^4 that normally exists across the plasma membrane, must be due to the mobilization of intracellular Ca^{2+} and cannot be due to the opening of a Ca^{2+} channel. It should be noted that excessive loading of the cells with quin2 can buffer this released Ca^{2+} and completely mask detection of the antigen receptor–mediated mobilization of intracellular Ca^{2+} (68). This property of quin2 is due to its ability to act as a Ca^{2+} chelator and has been explored in detail in several recent publications (69, 70).

Perturbation of T3/Ti must generate some signal that in turn releases intracellular Ca^{2+}. The putative Ca^{2+}-mobilizing signal for many receptors is inositol trisphosphate (IP_3), a water-soluble product of the hydrolysis of the phospholipid, phosphatidylinositol bisphosphate (PIP_2) (reviewed in 71). To determine whether perturbation of the antigen receptor complex generated IP_3, we labelled the membrane phospholipid pool of Jurkat cells with [^{3}H]-inositol (62). Following stimulation, the cells are lysed, the water-soluble fraction is obtained, and the inositol phosphates are separated by anion exchange chromatography. The addition of MAbs to either T3 or to Ti, but not to other cell-surface determinants (Leu 5, HLA

class I), generates substantial increases in IP_3, as well as in its breakdown products, inositol bisphosphate (IP_2) and inositol phosphate (IP_1) (62). Changes in IP_3 are detected within 20 sec after the addition of MAbs to T3/Ti (62). Therefore, the generation of IP_3, like the increase in $[Ca^{2+}]_i$, is an early and specific consequence of perturbation of T3/Ti. The increases in inositol phosphates, however, cannot be a consequence of the antigen receptor–mediated increase in $[Ca^{2+}]_i$ as ionomycin has no effect on the levels of any of these compounds (62). Purified IP_3 releases Ca^{2+} from intracellular pools in permeabilized Jurkat cells, suggesting that this compound functions as a Ca^{2+}-mobilizing signal in intact cells (62). The maximal amount of Ca^{2+} released by IP_3 from permeabilized cells is 100 pmol of Ca^{2+} per 10^6 cells (68). As perturbation of T3/Ti consistently releases 30 pmol of Ca^{2+} from the intracellular stores of 10^6 intact cells, the IP_3-accessible intracellular Ca^{2+} store can account for all of the T3/Ti-mediated mobilization of intracellular Ca^{2+} (68). Taken together, these results indicate that perturbation of the antigen receptor complex in turn activates a phosphodiesterase that generates IP_3 from PIP_2 (Figure 4). IP_3 is released into the cytoplasm where it mobilizes Ca^{2+} from a nonmitochondrial pool that is probably within the endoplasmic reticulum. This release of Ca^{2+} causes a prompt increase in $[Ca^{2+}]_i$ (Figure 4).

Mitogenic lectins also generate inositol phosphates when added to T lymphocytes. It has been known for several years that lectins increase the turnover of phosphatidylinositol (the parent compound of PIP_2) in peripheral-blood T cells (72). Recently, the addition of Concanavalin A to murine thymocytes has been shown to generate IP_3 and to mobilize intracellular Ca^{2+} stores (73, 74). We also have found that PHA and Con A generate substantial increases in all three inositol phosphates in Jurkat (68).

Although mobilization of intracellular Ca^{2+} accounts for the initial peak increase in $[Ca^{2+}]_i$ following perturbation of T3/Ti, the sustained increase in $[Ca^{2+}]_i$ requires the presence of extracellular Ca^{2+} (62). While the mechanism responsible for the sustained increase in $[Ca^{2+}]_i$ has not been elucidated, a classical voltage-gated Ca^{2+}-channel does not appear to be involved. Several groups, using very sensitive patch-clamping techniques, have failed to identify such Ca^{2+} channels in T lymphocytes (75, 76). Furthermore, we have observed that, in quin2-loaded Jurkat cells, the T3/Ti-mediated increase in $[Ca^{2+}]_i$ is not affected by the Ca^{2+}-channel blockers, nifedipine and verapamil, and that depolarization of the plasma membrane does not increase $[Ca^{2+}]_i$ (68). These observations, of course, do not exclude the possibility that T3/Ti activates a Ca^{2+} channel that differs fundamentally from the channels found on excitable cells, and they also do not rule out involvement of Ca^{2+} exchange mechanisms.

It is possible that the increase in $[Ca^{2+}]_i$ is sustained without increasing

the permeability of the plasma membrane to Ca^{2+}. We have recently observed that, 15 min after the addition of the Ti MAb to Jurkat cells in the presence of 1 mM extracellular Ca^{2+}, the level of IP_3 is still significantly elevated (68). Furthermore, intracellular Ca^{2+} stores remain depleted in these cells even though $[Ca^{2+}]_i$ has been maintained at a level greater than 200 nM for 15 min (68). These observations suggest that continued generation of IP_3, either by blocking reuptake of Ca^{2+} into intracellular stores or by continually depleting those stores, contributes to the sustained phase increase in $[Ca^{2+}]_i$. An additional mechanism that could sustain an increase in $[Ca^{2+}]_i$ is the slowing of the pumps that normally extrude Ca^{2+} from the cell in order to maintain the Ca^{2+} gradient across the plasma

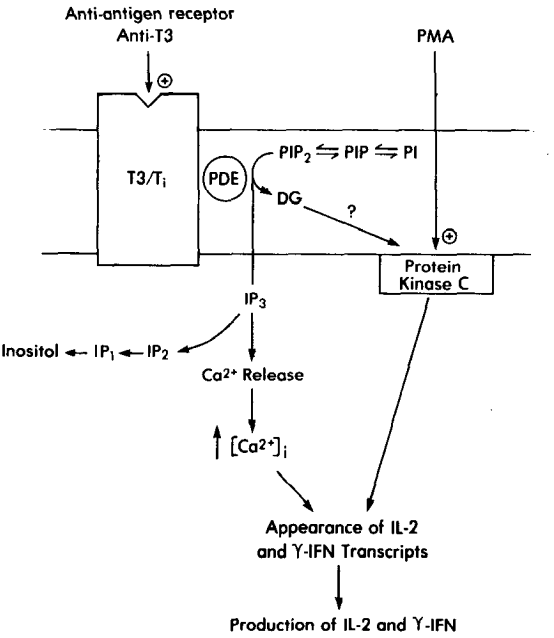

Figure 4 Model of the early events of antigen receptor activation. Perturbation of T3/Ti by monoclonal antibodies in turn activates a phosphodiesterase (PDE) that catalyzes the hydrolysis of the phospholipid phosphatidylinositol bisphosphate (PIP_2) to inositol trisphosphate (IP_3) and diacylglycerol (DG). IP_3 is released into the cytoplasm and mobilizes Ca^{2+} from a nonmitochondrial pool that is probably within the endoplasmic reticulum (14). This release of Ca^{2+} causes an increase in $(Ca^{2+})_i$. IP_3 is broken down to inositol bisphosphate (IP_2), inositol phosphate (IP_1), and finally to inositol which can be recycled into phosphatidylinositol (PI). DG is the putative physiological activator of protein kinase C (PKC). It is likely, therefore, that the perturbation of T3/Ti can activate PKC as well as increase $(Ca^{2+})_i$. Phorbol myristate acetate (PMA) also activates PKC (23, 24). Perturbation of T3/Ti and PMA act synergistically to induce the appearance of transcripts for IL-2 and γ-IFN and the production of IL-1 and γ-IFN biological activity.

membrane. Of note in this regard is the observation in hepatocytes that th Vmax of the plasma membrane Ca^{2+} pump is reduced by 50% followin vasopressin-induced release of Ca^{2+} (77). A similar mechanism may b operative following Con A–induced mobilization of intracellular Ca^{2+} i murine thymocytes (73). Finally, it should be emphasized that multipl different mechanisms may contribute to the sustained increase in $[Ca^{2+}]$ following perturbation of T3/Ti. Increased permeability of the plasm membrane to Ca^{2+}, the activation of Ca^{2+} exchangers, continued releas of Ca^{2+} from intracellular stores, and slowed Ca^{2+} extrusion are no mutually exclusive possibilities.

A TWO-SIGNAL MODEL FOR T-CELL ACTIVATION

In Jurkat cells, as well in other systems of T-cell activation, increases i $[Ca^{2+}]_i$ act synergistically with PMA to initiate the events that lead to th production of lymphokines (54, 58, 59). Synergism between these tw stimuli is not unique to T cells but appears to be a general mechanism tha can influence intracellular activities in a variety of different tissues (78 PMA has been shown to bind to, and to activate, protein kinase C (79, 80). I is a reasonable assumption that activation of protein kinase C accounts fo at least some, and perhaps all, of the effects of PMA on T-cell activatio Indeed, it can be demonstrated that PMA causes translocation of kinas activity from the cytosol to the plasma membrane of Jurkat (B. Manger, J Stobo, unpublished observations). Similar translocation has been used a an assay of protein kinase C activation in other systems.

The putative physiological activator of protein kinase C is diacylglycero a necessary product in the hydrolysis of PIP_2 to IP_3 (Figure 4) (71). The lin between T3/Ti and the generation of IP_3, therefore, ties T3/Ti to a pote transmembrane signalling system that has the capability to activate protei kinase C as well as to increase $[Ca^{2+}]_i$ (71, 78). Since we have shown tha MAbs to T3/Ti can hydrolyze PIP_2 to IP_3 and thus generate diacylglycero one might ask why these MAbs by themselves did not generate the tw signals (i.e. increases in $[Ca^{2+}]_i$ and protein kinase C activation) necessar for T-cell activation. Two possibilities might explain this. First, PMA migh provide a signal in addition to protein kinase C activation that is no provided by MAb to T3/Ti but that is necessary for activation. Secon while MAb to T3/Ti may induce the generation of diacylglycerol, the level may not be sufficient to activate protein kinase C.

When MAb to T3/Ti are immobilized by linkage to a solid surface suc as sepharose, they can activate T-cell clones in the absence of any additiona stimulus (7). Since clones have been previously stimulated, the basal state o activation may determine the quality or quantity of signals required for T

cell activation. This is supported by the following experiments. HUT-78 is another human T-cell line, which differs from Jurkat in that it displays the activation marker Ia (i.e. human HLA-DR) and receptors for IL-2. Therefore, Jurkat phenotypically resembles a resting and HUT-78-stimulated T cell. Activation of both Jurkat and HUT by MAb's to T3/Ti requires a second signal that can be mediated by PMA. MAbs to T3 immobilized onto sepharose or plastic petri dishes are not sufficient, by themselves, to induce IL-2 production by Jurkat. However, the same immobilized antibodies can, in the absence of any other added stimulus induce maximal IL-2 production by HUT-78. Immobilized antibodies to T3 can activate Jurkat if IL-1 is added. Our interpretation of these studies is the following. Activation of T cells that have not been previously stimulated requires at least three stimuli. One is represented by the binding of antibodies to appropriate epitopes in the T3/Ti complex. A second is represented by immobilization of these antibodies. A third is IL-1. The intracellular message for the first stimulus requires increases in $[Ca^{2+}]_i$. The intracellular message for the second and third stimuli is unknown. However, since PMA can induce IL-2 production by Jurkat when used in conjunction with soluble MAbs, it is able to substitute for the second and third stimulus. Therefore, the combination of anti-T3/Ti and PMA or calcium ionophores and PMA can provide the three stimuli necessary for activation of resting T cells represented by Jurkat. Others have postulated a three-signal requirement in the activation of T cells (81, 82).

Activation of previously stimulated T cells does not require IL-1. Immobilization of antibodies to T3/Ti allows perturbation of the antigen receptor complex to provide the proper intracellular signals, thus obviating the need for PMA. Presently we are assaying protein kinase C activation in HUT following the addition of soluble vs immobilized T3 antibodies.

Molecular Events in T-Cell Activation

Recently, both IL-2 and IFN-γ genes have been cloned, and cDNA probes for each made available. Therefore, it is possible to explore the level—i.e. gene transcription, RNA processing, translation—at which signals that activate T cells influence the expression of lymphokine genes such as IL-2 and IFN-γ. In the model represented by activation of Jurkat, it is possible that antibodies to T3/Ti exert their effect at one level of gene expression such as transcription while PMA influences another such as RNA processing. Alternatively, both stimuli may be required for maximal activity at a single step in gene activation such as transcription. This section examines the data relating to these possibilities.

Other investigators have demonstrated that nonstimulated, peripheral-blood T cells, tonsilar lymphocytes, and cell lines such as Jurkat do not

contain transcripts (i.e. mRNA) hybridizing with cDNA probes for either IL-2 and IFN-γ (83, 84). When these same populations are appropriately stimulated, abundant transcripts from both genes are detectable. Therefore, activating signals cause expression of IL-2 and IFN-γ genes by influencing events that occur prior to the translation of mRNA. These events include gene transcription and processing of the primary RNA transcript.

To determine more precisely how T-cell activation leads to gene expression Kronke et al performed nuclear "run-on" experiments (84). This procedure utilizes radioactive RNA precursors and endogenous nuclear polymerases to label native nuclear RNA in vitro by elongating incomplete, nascent RNA transcripts previously initiated in vivo. Artifacts common to complete in vitro transcription systems are thus avoided. After purification, these labeled transcripts are hybridized to specific immobilized recombinant DNA gene fragments. The extent of hybridization reflects primarily the transcriptional activity of a particular gene or gene region. These experiments demonstrated that in nonstimulated Jurkat there is no detectable transcription of the IL-2. In contrast, when Jurkat is stimulated with both PHA and PMA, nuclear transcripts are detected. These studies did not examine nuclear transcription occurring after stimulation with either PHA alone or PMA alone. The most straightforward interpretation of these experiments is that in a two-stimulus model of T-cell activation, both stimuli exert their effect at the level of gene transcription. However, in the run-on experiments the recovery of the nuclear transcripts depends not only on the rate of transcription but also on the degradation of the RNA. Therefore, it is possible that one or both signals influence the level of detectable IL-2 transcripts by slowing RNA degradation rather than by increasing the rate of transcription.

We have used cDNA probes for IL-2 and cDNA, as well as genomic probes for IFN-γ, to examine molecular events involved in expression of these genes. Initial studies demonstrated that nonstimulated Jurkat did not contain any detectable IL-2 or IFN-γ-specific RNA. Stimulation of Jurkat with PHA alone, anti-T3 alone, or PMA alone failed to induce the appearance of either IL-2 and IFN-γ RNA. In contrast, the combination of PHA plus PMA on anti-T3 plus PMA did generate detectable transcripts for both genes. This did not represent simply a general increase in RNA occurring with activation since there was no substantial increase in actin-specific RNA during activation. These results are consistent with those of other workers and exclude the possibility that activating stimuli influence posttranslational events. Instead, the data suggests that they modify either gene transcription or RNA processing.

We have recently used a nuclear transcription assay such as that used by Kronke et al in combination with specific IFN-γ probes to localize more

precisely the site at which these signals influence gene expression. When compared to nonstimulated Jurkat, IFN-γ nuclear transcripts increase fourfold after stimulation with PHA and PMA; this suggests that both stimuli modulate gene transcription (Figure 5). That actin RNA is not affected by activation minimizes the possibility of effects on global RNA stability as an explanation of this observation. However, since detection of nuclear transcripts in the run-on assay requires that the transcript be protected from degradation, the possibility that both signals specifically affect stabilization of IFN-γ transcripts within the nucleus cannot be excluded. As we discuss subsequently, there is data that indirectly supports the hypothesis that transcriptional activity exists for at least one of the two signals required for activation.

Given the limitation of the run-on experiments in distinguishing precisely

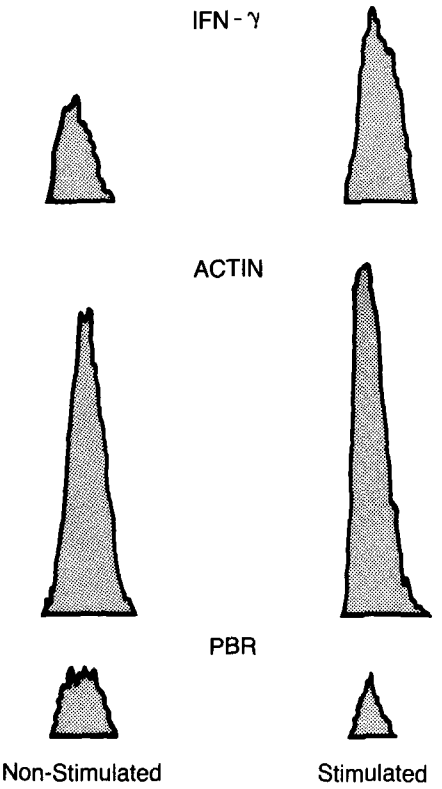

Figure 5 Nuclear transcription in nonstimulated and stimulated Jurkat. Nuclear run-on experiments were performed with cDNA probes for IFN-γ, actin, and PBR-322 using nuclei isolated from nonstimulated Jurkat and from Jurkat stimulated with PHA and PMA. The results are presented as densitometric tracings of dot blots.

transcriptional vs processing events, another approach was used to localize more precisely the level at which activation signals influence gene expression. This approach takes advantage of the fact that the conformation of regions of a gene that regulate transcription is such that they are unusually sensitive, i.e. hypersensitive, to digestion with pancreatic deoxyribonuclease (DNAase). For example, activation of the chicken globin gene with specific inducing agents results in the appearance of a new DNAase-hypersensitive site in the 5′ region (85). Hormonal induction of chicken lysozyme in vitallogenin genes is accompanied by the appearance of several new hypersensitive sites (86). Inactivation of fetal hemoglobin genes results in the loss of hypersensitive sites (87). Hypersensitive sites present in κ light-chain and IgM heavy-chain immunoglobulin genes can be directly implicated in the regulation of immunoglobulin gene transcription (88).

We have used this approach to study further the γ-IFN gene and have identified three major regions of strong DNAase-I hypersensitivity that correlate with regulation of that gene (Figure 6). One site localizes to the first intron and is found only in cells potentially capable of synthesizing γ-IFN. In other words, this intronic-hypersensitive site is seen in T cells capable of synthesizing γ-IFN but not in other cells such as B cells, monocytes, and nonlymphoid cells. This hypersensitive site is present in nonstimulated, as well as PHA/PMA-stimulated, Jurkat, which indicates that it is not an "activation-specific" regulator of transcription. Another area of hypersensitivity is localized just 5′ to the site for initiation of transcription. This site is in close proximity to the γ-IFN gene promoter. This area is insensitive to DNAase I in unstimulated Jurkat but develops hypersensitivity after stimulation with PHA and PMA. A third area of

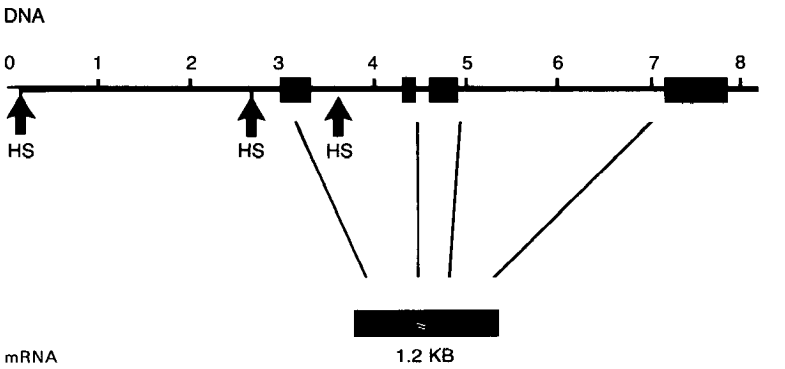

Figure 6 DNAase hypersensitivity sites in Jurkat, the IFN-γ gene. The three major areas of DNAase hypersensitivity located in the IFN-γ gene are presented. The numbers indicate KB and solid boxes the exons.

hypersensitivity is found in unstimulated Jurkat, 3 kb 5′ to the first exon. This site increases in hypersensitivity after PHA/PMA stimulation. To this point, changes in hypersensitivity in the 5′ sites have not been assayed after stimulation with a single activational signal (i.e. PHA or PMA alone).

We interpret these experiments in the following way. At least three regions of the γ-IFN gene are involved in regulating its experesion. One resides in the first intron, and an appropriate chromatin confirmation at this site seems necessary but not by itself sufficient for γ-IFN transcription. Two other sites are in the 5′ flanking region of the gene, and these either appear or markedly increase only after T-cell activation. These observations of induced hypersensitivity further support the notion that signals required for γ-IFN gene activation indeed exert their effect at least in part at the level of gene transcription. Preliminary experiments are under way to determine how cell-surface signals of activation are transduced into gene activation at this level by investigating binding of specific regulatory proteins to these hypersensitive regions of DNA in stimulated and unstimulated cells. It is likely that synthesis and/or modification of such proteins is somehow regulated by the signals inducing γ-IFN gene activation.

A kinetic analysis of the appearance of the IL-2 and γ-IFN mRNA vs biologic activity after stimulation of Jurkat with PHA and PMA reveals the following (Figure 7). Transcripts for both lymphokines are first detectable 2 hr after stimulation, peak at 4–6 hr, and then decline so that by 24 hr they have approached basal levels seen in nonstimulated cells. Biologic activity for both lymphokines is first detectable 4 hr after stimulation, with maximal amounts found in cell culture fluids 24 hr after activation. This decline in detectable RNA could reflect cessation of transcription or increased degradiation of transcripts. Efrat & Keampfer demonstrated that addition of cyclohexamide, an inhibitor of protein synthesis, to mitogen-stimulated tonsillar lymphocytes leads to a marked and sustained increase in IL-2 mRNA (83). This "super induction" of IL-2 transcripts was inhibited by 5,6-dichloro-1-beta-D-ribofuranosyl benzimidazole, a compound that inhibits gene transcription. This suggested that accumulation of mRNA seen following the addition of cyclohexamide was dependent on continued gene transcription and implied that the decline normally seen in IL-2 transcripts following T-cell activation reflects the induction of repressor molecules requiring new protein synthesis. In studying the same phenomena, Kronke et al arrived at a slightly different conclusion (84). In a series of "run-on" experiments these investigators noted that nuclear transcription of IL-2 continues for at least 15 hr following stimulation of Jurkat at a time when mRNA levels are decreasing. Therefore, they concluded that nontranscriptional regulatory mechanisms are involved in the rapid fall of IL-2, and

presumably γ-IFN transcripts, following activation. Such nontranscriptional regulatory mechanisms could include degradation of the transcribed RNA. Since cyclohexamide has also been reported to inhibit degradation of RNA, the results of Efrat & Keampfer could be partially consistent with the interpretation of Kronke and his colleagues. In other words, as a late response to the increased transcription of IL-2 and γ-IFN induced by activating signals, there seems to occur a negative feedback system by which repressor-induced reduction in transcription and/or increase in selective RNA degradation can lower intranuclear transcripts of these lymphokine genes. Either of these pathways if inhibited by cyclohexamide could result in prolonged appearance of transcripts. There are numerous examples of

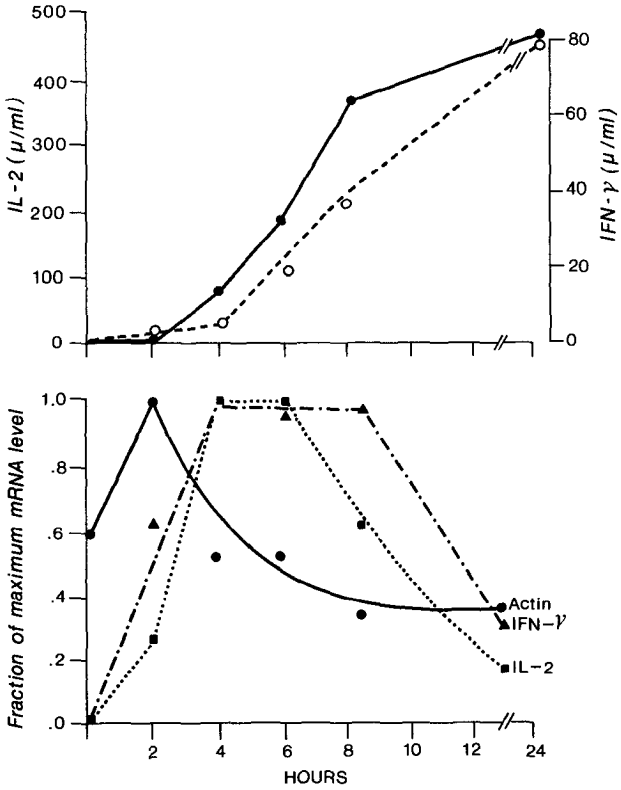

Figure 7 Appearance of IL-2 and IFN-γ activity and mRNA in Jurkat. Jurkat was stimulated with PHA and PMA. At the indicated times IL-2 activity (solid line, top) and IFN-γ activity (dotted line, top) were measured and are presented as units/ml. Total RNA hybridizing with cDNA probes for actin, IL-2, and IFN-γ were also assayed by dot blots with the results presented as a fraction of the maximal amount of hybridizing RNA as determined by densitometric analysis.

gene repressors in prokaryotic systems and several new lines of evidence that eukaryotic repressors of gene transcription do indeed exist although they are certainly less well elucidated. Further study of the molecular mechanisms that result in the turning on and off of IL-2 γ-IFN genes will be particularly important to our understanding of T-cell activation, lymphokine gene regulation, and overall immune responsiveness.

SUMMARY

The role of the T3/antigen receptor complex is summarized by the diagram presented in Figure 4. Signals transmitted through T3/Ti activate a phosphodiesterase. This enzyme acts on its substrate PIP_2 to generate two important mediators, IP_3 and diacylglycerol. IP_3 mobilizes calcium from bound intracellular stones. This increase in $[Ca^{2+}]_i$ is one intracellular signal which, in conjunction with others, induces expression of lymphokine genes by influencing pretranslational, presumably transcriptional, events.

Several problems remain. Which of the five molecules in the T3/Ti complex serves as the effector molecule in the transmembrane signalling process is not known. Which molecules serve to link T3/Ti to the phosphodiesterase enzyme is under investigation. The role diacylglycerol protein kinase C and other mediators play in signalling activation is not established. Finally, for those events occurring after the early events pictured in Figure 4 that result in gene activation, the sequence is a black box. Approaches to address each of these questions are available, and answers should be forthcoming.

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CONTENTS

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THE PHYSIOLOGY OF B CELLS AS STUDIED WITH TUMOR MODELS

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INTRODUCTION

An exquisite adaptation of vertebrates appears in their production of antibodies in response to infectious agents. When antibodies bind to microbes in vivo, they trigger cascades of events that typically eliminate the infectious agent. The degree to which the relevant antibodies are expressed during such an immune response is a function of both the size and stage of differentiation of specific B-cell clones. The factors that influence these parameters are multiple, complex, highly regulated, and the focus of much current research.

During the past 20 years, our knowledge of antibodies has grown extraordinarily. Much has been learned about their molecular structure, the chemical basis of their binding specificity, the structural basis of their idiotypes, allotypes, and isotypes, and the structure and organization of their genes (1). Virtually all of these advances in molecular immunology derive from studies with B-cell tumors. The present review considers recent studies that have successfully used B-cell tumors to investigate some of the physiological processes that underlie B-cell function and regulation.

A major advance in the study of antibodies was the observation reported in 1967 by Eisen et al (2) that human myeloma proteins were, in fact, monoclonal antibodies. This finding was rapidly confirmed for murine myeloma proteins by Eisen et al (3) and Schubert et al (4). The discovery that murine myeloma proteins were monoclonal antibodies was particularly significant because a sizable library of BALB/c plasmacytomas

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had been developed by Michael Potter and colleagues at the National Cancer Institute. These monoclonal immunoglobulins were systematically screened for antibody activity, and some were identified that specifically bound dextrans, levans, phosphorylcholine, 2,4-di-nitrophenyl, or other ligands (5).

The identification of myeloma proteins with antibody activity was an exciting finding that came at a time when efforts to investigate the structural basis of antibody binding had reached an impasse because of the molecular heterogeneity of conventional antibodies. The availability of large quantities of antigen-binding myeloma proteins led immediately to structural studies. The subsequent development of hybridomas by Kohler & Milstein (6) made possible isolation of monoclonal antibodies from virtually anywhere in the B-cell repertoire and extension of the structural studies to antibodies of any desired antigen specificity and isotype. While the original objective was to establish the structural basis of antibody binding, the wave of amino acid sequence analyses of murine myeloma proteins in the 1970s had the additional impact of generating data that began to (*a*) raise questions about genetic and somatic mechanisms that might account for the enormous size and diversity of the antibody repertoire, and (*b*) provide clues to the organization of the genetic elements that coded for immunoglobulin chains. The history of these developments is detailed by Kindt & Capra (1).

In addition to their dominant role in the development of knowledge about antibody structure, B-cell tumors have been the major source of new information about immunoglobulin genes. The pioneering studies of Hozumi & Tonegawa (7) used murine plasmacytoma cells to begin to visualize the molecular organization of immunoglobulin genes and to identify the developmental rearrangements that occured in these genes in immunoglobulin-expressing cells. Molecular genetic tools and strategies have been successfully applied to murine plasmacytoma cells to investigate (*a*) the nucleotide sequences, organization (8), and chromosomal locations (9) of immunoglobulin genes; (*b*) the role of RNA processing in the expression of immunoglobulin genes; and (*c*) the structural differences between surface membrane and secreted immunoglobulin heavy chains (11). Furthermore, murine plasmacytoma cells have been productively used: (*a*) to identify steps in immunoglobulin chain synthesis and assembly (12), (*b*) to investigate posttranslational modifications of immunoglobulins (13), and (*c*) to assess the role of glycosylation in immunoglobulin secretion (14).

From the material briefly reviewed above, it is clear that B-cell tumors have been extremely useful tools for the study of immunoglobulins and their genes as they exist in a more or less fixed state in each clone. The

experimental strategy common to these studies has been the utilization of tumor cells as the source of a single, specific, readily purified macromolecule such as an antibody, a messenger RNA, or an immunoglobulin gene. The present review discusses recent studies that establish the feasibility of using B-cell tumors to investigate dynamic aspects of B-cell function such as immunoglobulin gene activation (15), heavy-chain class switching (16), and regulatory mechanisms that control B-cell proliferation, differentiation, and immunoglobulin expression (17). Progress made in many laboratories during the past five years has shown that B-cell tumors are powerful model systems that can be productively used to investigate physiological processes in B cells and to develop an understanding of the mechanisms by which immunoregulatory signals influence B-cell function.

RELEVANCE OF TUMOR MODELS

An important issue to address at the outset is the relevance of observations made with tumor cells to an understanding of the physiology of normal B cells. This question is frequently raised because certain characteristics of tumor cells are considered aberrant. The designation "aberrant" carries with it the implication that the feature is a curiosity of the tumor state, is of no physiological relevance, or has no normal counterpart. However, many "aberrant" features of tumor cells have later proved to be normal features that had previously gone undetected. An example is the expression of Lyt-1 by some B-cell tumors (18). Lyt-1 was considered to be a T cell–specific marker. Its "aberrant" expression on some malignant B cells preceded its detection on a subpopulation of normal B cells (19). The prophetic nature of some aberrant features is not restricted to lymphoid tumors. For example, syndromes of hormone excess associated with carcinomas of the lung and gastrointestinal tract were originally considered aberrant expressions of "de-repressed" polypeptide hormone genes in malignant mucosal cells. In retrospect, however, these tumors facilitated discovery of the intestinal and bronchial endocrine systems by pointing out a previously unknown population of normal mucosal cells that produce these hormones. These examples emphasize that an unknown fraction of "aberrant" features of tumor cells have normal counterparts that simply have not yet been discovered. Considered in this sense, tumors do not lie; they only exaggerate.

Perhaps the greatest value of tumor models comes from investigations of bona fide aberrant features. For example, when the amino acid sequences of the truncated heavy chains produced by lymphoma cells in Franklin's disease were compared with the normal-sized heavy chains of human myeloma proteins, the data implied that the DNA that coded for the

variable region domain was separated by a significant distance from the DNA that coded for the constant region (20). This finding was one of the first pieces of evidence in confirmation of the Dryer-Bennett hypothesis that two genes encoded a single polypeptide chain (21). DNA sequence analysis later proved this predicted pattern of immunoglobulin gene organization to be correct.

In summary, the appropriateness of using tumors as models might best be considered in terms of the enormous productivity and success of this approach. It is difficult to find examples in which information obtained from studies with tumor cells has been misleading. In fact, one could argue that tumor cells can be more useful models than normal cells themselves because of the extraordinary insight that can be prompted by an aberrant feature. The study of tumor cells to develop an understanding of normal cells is a specific instance of the more general and proven principle that the study of pathological processes often leads to an improved understanding of the normal state. Perhaps nowhere is this more true than in immunology, where the study of immunodeficiency diseases has provided so much insight into the development, organization, and function of lymphoid cells and tissues.

THE DEVELOPMENT AND COMMITMENT OF B CELLS STUDIED WITH PRE–B CELL TUMORS

Until recently, the earliest stages of differentiation during B-cell ontogeny have been hard to define because they are transient, and in most lymphoid tissues, cells in these stages represent only a minor component of a widely diverse population. Furthermore, immature B cells and B-cell precursors are not functionally competent and thus cannot be studied using classical techniques that assay for activation or antibody production. In fact, their existence was deduced from experiments in which polyclonal explants of murine fetal liver or adult bone marrow were found to generate populations of cells bearing surface immunoglobulin (sIg) after several days of culture in vitro (22). Using immunofluorescent techniques and unit gravity sedimentation, researchers demonstrated the existence of populations of both large and small pre-B cells expressing cytoplasmic μ–heavy-chain protein, but not sIg or light-chain protein, which might give rise to B lymphocytes expressing surface IgM (23–27). Such cells were found to lack Fc and C3 receptors as well as Ia determinants (28, 29), which are displayed by mature B cells.

Initial attempts to culture or clone sIg^- precursors for further characterization were disappointing. A major breakthrough in this regard came

when it was realized that the Abelson murine leukemia virus (A-MuLV) would preferentially transform cells with a pre-B phenotype (30). In addition, a murine leukemia cell line, 70Z/3, which had been induced by methyl nitrosourea, was characterized as having a pre-B phenotype (31). Isolation of these tumors spurred a reexamination of lymphomas that, because they exhibited neither immunoglobulin synthesis nor the surface antigens of T cells, were thought to be unusual neoplasms. Analysis of these lymphomas, as well as of some human non-T, non-B actue lymphocytic leukemia (ALL) cells, demonstrated that many of these have the characteristics of B-cell precursors (32, 33). In addition, some cell lines transformed with Epstein Barr virus (EBV) displayed a pre-B phenotype (34), as did fetal liver hybridomas (35). The availability of these cell lines has had a profound impact on studies of the processes involved in B-cell differentiation. They have provided useful homogeneous populations of cells in numbers sufficient for functional and biochemical analyses and so have allowed investigators to address problems previously inaccessible.

Analysis of Cell-Surface Antigens of B-Lineage Precursors

One major problem in studying B-lymphocyte precursors has been identification. Although many differentiation antigens specific for mature B cells have been described, until recently detection of cells with the pre-B phenotype relied primarily on the presence of cytoplasmic μ–heavy chain in the absence of sIg and other differentiation antigens. Lately, several laboratories have addressed this problem and, using human- and murine-transformed cell lines as immunogens, have generated a number of monoclonal antibodies (MAb) that recognized cell-surface antigens on normal pre-B cells from bone marrow and fetal liver. These are summarized in Table 1.

Although these antibodies react with surface antigens on cells that express a pre-B cell phenotype, many also bind to sIg^+ B cells (14.8, DNL1.9, GF1.2, BA-2) or plasma cells and plasmacytomas (RA3-2C2). BA-2 and J-5 bind to some human T-ALL cells, while $AA4.1^+$ cell populations include erythroid and myeloid progenitors. However, with respect to B-lineage lymphocytes and transformed cells, the AA4.1 and GF1.2 appear to be specific for antigens expressed in the early stages of differentiation (36). In combination with previously characterized reagents, these MAb are proving useful in identifying and selecting subsets of B lymphocytes. Recently Paige et al (43) have used AA4.1 and 14.8 to clone pre-B cells from normal fetal liver or bone marrow and to analyze their responsiveness to growth factors. The MAb 14.8 also has been used by Jyonouchi et al (44) to

Table 1 Monoclonal antibodies that recognize cell-surface antigens on normal pre-B cells from bone marrow and fetal liver

MAb	Produced in	Immunogen	Reference
AA4.1	Rat	70Z/3	36
GF1.2	Rat	ABE-8	36
RA3-2C2	Rat	RAW-112	37
DNL1.9	Rat	70Z/3	38
14.8	Rat	WEHI-279	39
$\bar{\alpha}$ BL1	Mouse	Nalm-6	40
BA-2	Mouse	Halm-6MI	41
J-5	Mouse	Human ALL cells	42

study the age-dependent immunodeficiency of New Zealand black (NZB) mice, while J-5 and BA-2 have been used to phenotype acute lymphoblastic leukemia (ALL) cells prior to further biochemical analysis (45).

Immunoglobulin Genes in Pre-B Cells: Structure, Rearrangement, Expression, and Regulation

The availability of transformed cell lines exhibiting a pre-B phenotype allowed analysis of the rearrangement of immunoglobulin genes that occurs during B-cell ontogeny, with some stunning results. A variety of A-MuLV or EBV transformants, as well as fetal liver hybridomas and ALL cells, were useful in this regard, exhibiting characteristics intermediate to those described for embryonic tissues and mature B lymphocytes. Moreover, since some of these cells—70Z/3 and 18–81 (31, 46)—can be induced to differentiate, the sequence of events leading to Ig-gene expression and the regulation of that expression could be addressed.

STRUCTURE OF Ig GENES: AN OVERVIEW Immunoglobulin (Ig) genes undergo rearrangements in a tissue-specific manner. This became apparent after the organization and structure of these genes in Ig-producing cells were compared to their configurations in embryonic and nonlymphoid tissue. These complex genes are found in three distinct loci on separate chromosomes, and as is true for many eukaryotic genes, coding regions for both heavy- (IgH) and light-chain (IgL) immunoglobulin genes are segmented. The germline organization and structure of murine and human immunoglobulin genes have been described in detail in a recent review (47) and are discussed only briefly here. In mice, all of the coding regions (exons) for heavy-chain genes are located on chromosome 12 and arranged so that segments encoding variable regions of Ig protein are separated from constant region exons. The sequences that code for variable regions of Ig

protein are subdivided into three groups or families of segments for the heavy-chain genes (V, D, and J) and two for each of the light-chain genes (V and J). These groups are arranged sequentially with between 100 and 200 variable (V_H) region exons at the 5′ end, followed in order by at least 14 diversity (D) elements unique to heavy-chain genes, and 4 functional joining (J_H) region exons (as well as 1 J_H pseudogene). The constant region genes for each of the 8 heavy-chain isotypes lie at the 3′ end of this locus. Murine κ light-chain genes are organized similarly, on chromosome 6, with an estimated 280 V_κ gene segments, 4 J_κ segments, and a single κ–constant region (C_κ) exon. The murine λ–light chain genes, found on chromosome 16, are organized somewhat differently, with only 2 variable region (V_λ) segments but 4 J_λ– and λ–constant region (C_λ) genes. These are arranged so that 2 J_λ-C_λ pairs lie in tandem behind each V_λ gene (i.e. V_λ-J_λ-C_λ-J_λ-C_λ). Human Ig loci have basically the same structure and organization, differing from murine loci in the DNA sequences of coding regions and in the number of elements found in each of the V, D, and J families. In the embryonic configuration, each of these elements and each family of segments is flanked by intervening sequences (IVS) of noncoding DNA, often several kilobases (kb) in length. However, DNA sequencing of variable region Ig genes in myelomas and plasmacytomas showed that transcriptionally active genes are rearranged in such a way as to juxtapose one element from each family of variable region coding sequences (V_H, D, and J_H at IgH loci, and V_L and J_L at IgL loci), with concomitant removal of any DNA that had separated these sequences in germline configuration. From this, it became obvious that B-cell precursors must rearrange IgH and IgL genes as part of the differentiation process. A detailed analysis of the reorganization of Ig genes was made possible by the availability of homogenous populations of transformed B-cell precursors and demonstrated that recombination at Ig loci occurs in an ordered sequence of discrete steps.

REARRANGEMENTS OF Ig GENES IN TRANSFORMED PRE-B CELLS The reorganization of Ig gene loci is discussed in detail in a review in this volume (48) and so is only summarized briefly here. Reorganization of the Ig genes begins at the IgH loci with a D-to-J_H recombination. The DJ_H complex then serves as an intermediate for V_H to DJ_H rearrangement. The V_H-to-DJ_H recombination occurs on one allele at a time, and if the first rearrangement is productive, the second allele remains in the intermediate configuration. If the first V_H-to-DJ_H rearrangement is aberrant or nonproductive, the second allele can be rearranged, as described by Alt et al (49) for cells of murine origin and by Korsmeyer et al (45) who used human non-T, non-B ALL cells. Thus, a secondary or V_H-to-DJ_H recombination does not in itself

appear to provide a signal sufficient to stop IgH gene rearrangements. The rearrangement must also be "productive" in that a protein is synthesized from the processed transcripts. Otherwise, rearrangements will continue, as evidenced by the frequency of aberrant V_H-to-DJ_H rearrangements identified so far (49) in a variety of cell lines. It is interesting to note that no V_HD or DD segment rearrangements have been detected in any of the cell lines studied so far. Also of interest is the observation that when both IgH alleles had V_H-to-DJ_H rearrangements, at least one of them was aberrantly rearranged and so would yield no heavy-chain protein.

Only after a productive IgH rearrangement has been made can recombination be detected at IgL loci. Both normal B cells and their transformed analogues frequently exhibit rearrangements of more than one IgL allele and/or locus (50–56). Furthermore, productive rearrangements at IgL loci must provide not only a light-chain protein, but also one that can combine with the μ heavy chain already expressed, in order to generate a functional immunoglobulin protein. It can be argued that synthesis of κ-chain protein somehow provides a necessary signal for cells to stop rearranging IgL loci. In the absence of such a signal, recombination can continue, with the possibility of generating a productive gene at either an unrearranged allele or at an aberrantly rearranged locus (56, 57). To date initial rearrangements most frequently involve κ- rather than λ-gene loci, and this suggests a hierarchy of light-chain gene rearrangements found in normal B cells and their transformed counterparts, as well as asynchrony in heavy- and light-chain gene rearrangements. Each of the recombinatorial events described above was defined initially in transformed cell lines and subsequently confirmed in studies of normal cells, which underscores the utility of tumor models in delineating B-cell physiology at the molecular level.

IMMUNOGLOBULIN GENE EXPRESSION IN PRE B–CELL MODELS Apparently, transcription of recombined Ig genes and translation of their mRNAs into proteins can occur as soon as a productive recombination has been made. Thus, μ–heavy chain protein appears in the cytoplasm of cells that have not yet rearranged their light-chain genes (58, 59, 56, 57). Remarkably, IgH genes in the DJ_H intermediate configuration also are transcribed (59, 60). Studies of 12 bone marrow–derived and 10 fetal liver–derived A-MuLV transformants by Reth & Alt (59) addressed this phenomenon directly, demonstrating that (*a*) while one allele was transcribed in some of the cell lines they studied, others were producing mRNA from both alleles; (*b*) subsequent V_H-to-DJ_H rearrangements, which occurred spontaneously and rather frequently in fetal liver-derived lines, could generate a functional and actively transcribed gene; and (*c*) both alleles may continue to be

transcribed, even after a productive V_H-to-DJ_H rearrangement. Again, in all of these cell lines, the IgL loci were in germ-line configuration.

Not only are the DJ_H loci transcribed, but synthesis of protein with Cμ sequences also can occur as soon as a productive DJ_H-intermediate has been formed. Translation of such an RNA generates a truncated μ–heavy chain, or Dμ, protein. Reth & Alt (59) demonstrated that several of the A-MuLV transformants containing only DJ_H rearrangements produce Dμ protein. It had been demonstrated previously that secretory and membrane-associated μ-chains are translated from mRNAs that differ only at the 3′ end (10, 61), this alteration results from differential splicing of primary transcripts from the same gene (10). As a result two polypeptides are synthesized that differ in their carboxyterminal sequences (11, 62), i.e. both secretory and membrane-bound forms of these Dμ proteins might be synthesized. However, while protein containing heavy-chain sequences can be detected in the cytoplasmic compartment, as yet no Dμ protein has been found on the surface of these A-MuLV transformants, even though the predicted amino acid sequences of Dμ proteins synthesized by these cell lines include amino terminal sequences that might serve as signal peptides for translation of their mRNAs on membrane-bound polyribosomes (63).

Another interesting set of observations has been made regarding these gene products. Previously, Perry & Kelley (64) had reported the isolation of four mRNAs containing Cμ sequences, three of which—3.0, 2.7, and 2.4 kb in length—are associated with membrane-bound polyribosomes and one of which was found predominantly in free (cytoplasmic) polyribosomes and in ribonucleoprotein of 70Z/3 cells. This mRNA was 2.1 kb long and was not found in WEHI 231 cells, which exhibit a more mature phenotype; these cells were shown to contain only the other three sizes of Cμ-encoding mRNAs. By comparison, Northern analysis of the Cμ-encoding mRNA from pre-B cell hybrids derived from patients with x-linked agammaglobulinemia (XLA) showed that these cells also contain mRNAs 2.0–2.1 kb long, roughly 300 bases shorter than the μ-chain mRNA from SM14, a normal B-cell line (65). Taken together, these observations suggest that the 2.1-kb mRNA from 70Z/3 might encode a truncated (Dμ) heavy chain derived from the IgH allele containing only a DJ_H rearrangement, which is not translated on membrane-bound polyribosomes. Thus, the protein could be neither expressed on the plasma membrane, glycosylated, nor secreted. If this is the case, then concurrent transcription can take place on both IgH loci in 70Z/3 cells, as had been observed by Reth & Alt (59) in A-MuLV-transformed cell lines. Furthermore, expression of Dμ transcripts might be developmentally regulated so that Dμ protein is synthesized only in cells that do not make light-chain protein.

In support of the physiological relevance of these observations, Schwaber

et al (65) noted that approximately 5% of normal pre-B cells from human adult bone marrow produced polypeptides of 54–57 kilodaltons (kd) containing μ–heavy chain determinants, but lacking a V_H region. The pre-B cells from patients with XLA were all found to have this phenotype, suggesting the XLA might result from a block in differentiation of B-cell precursors at the second step in IgH gene rearrangement. Similar results were obtained by Levitt & Cooper (66), who demonstrated not only synthesis, but also secretion of short μ–heavy chains of sIg^- cells from murine fetal liver cells, as well as those from a patient with XLA. It has been suggested that these polypeptides might serve some regulatory function, but such a role is purely speculative at this point.

REGULATION OF Ig GENE EXPRESSION DURING EARLY B-CELL DEVELOPMENT Transformed precursor cell lines also have provided model systems for studying the regulation of gene expression during differentiation. A mature B lymphocyte produces a single functional immunoglobulin molecule containing at least two identical heavy chains and two light chains. Each type of polypeptide is the product of a single rearranged gene. In mature B cells and their transformed analogues, transcripts from productively rearranged genes predominate among the variety of possible transcripts (64, 67), implying that expression of these genes is controlled at least partially at the level of transcription. In support of this notion, a transcriptional enhancer region has been identified that operates in a tissue-specific manner (68, 69). This enhancer is found between J and constant region exons in both heavy-chain and κ–light chain genes (69, 70). In 70Z/3 cells, this region of the κ–light chain locus becomes hypersensitive to nuclease digestion after treatment with LPS, which induces transcription of the rearranged κ gene (71), in a cell cycle–dependent manner (72).

However, one subclone of 18-81 (an A-MuLV transformant) expresses high levels of cytoplasmic μ–heavy chain despite deletion of the enhancer element on the transcribed allele (73). Yet this cell line is unique, since most μ^+, light chain–negative Abelson transformants express little heavy-chain protein (46, 74). A high intracellular level of heavy-chain protein in the absence of light chain appears to be toxic, since it adversely affects growth in plasmacytomas that have lost light-chain gene expression (75). However, variants of these cells can be isolated that continue to synthesize abnormal heavy-chain protein (76, 77). Such an effect may be at work in the 18-81 parental line, because μ-protein synthesis decreases during long-term culture of these cells, and subcloning of this cell line isolated variants carrying deletions that removed some or all of the enhancer region. These cells produced very low levels of heavy-chain mRNA and protein, but both transcription and translation could be induced by treatment with LPS;

this only affected expression of alleles containing productively rearranged variable regions (78).

Some of these secondary subclones appear to have undergone class switching (79–81), an event known to occur in mature B cells. Class switching results in expression of a heavy-chain isotype other than μ and is associated with deletion of huge stretches of DNA containing both coding and noncoding regions. The sequences of heavy-chain genes generated by this kind of rearrangement have been studied in a large variety of plasmacytomas and myelomas; such sequences uniformly involve recombination sites called switch regions (reviewed in 47 and 82). However, the apparent class switching seen in Abelson transformants does not meet these criteria and so may not be physiologically relevant in this regard. Instead, the variants that have been isolated may be deletional mutants selected as a function of clonal outgrowth.

By definition, pre-B cells do not synthesize light-chain protein or express sIg. These events appear to be coupled, in that sIg appears as soon as synthesis of light-chain peptide can be detected. The resulting change in phenotype is associated with differentiation of precursors into immune competent cells, i.e. with a pre-B to B-cell transition. As mentioned previously, 70Z/3 cells have one productively rearranged κ-gene locus and constitutively produce low levels of μ–heavy chain. When treated with LPS (31), IL-1 (83), or BMF (84), they synthesize κ–light chain protein and begin to express surface IgM, suggesting a transcriptional level of control for expression of light-chain protein in 70Z/3. However, despite high levels of secretory μ–heavy chain protein in these cells, they never secrete IgM (84, 85). Since these cells do not make J chain, this lack of secretion may result from an inability to assemble the IgM pentamer. These cells appear to represent precursors frozen at a stage of differentiation immediately preceding the pre-B to B-cell transition but capable of responding to immunoregulatory stimuli by synthesizing light-chain protein. 70Z/3 cells appear to be unique in this regard, since other cell lines that have productively rearranged an IgL locus synthesize light-chain protein constitutively (62). Investigations of 70Z/3 cells have provided many interesting insights. Studying these cells, Parslow et al (15) defined another putative control region. They identified a highly conserved sequence of eight nucleotides, positioned approximately 70 base pairs upstream of the transcriptional initiation site of the rearranged κ gene in 70Z/3 and 13 other light-chain genes (both murine and human). A precise inversion of this sequence is found in the same position in heavy-chain loci. No homologies exist in sequences flanking this octanucleotide. Since both sequence and location are highly conserved, this locus may serve as a site of recognition for factors regulating Ig gene expression. Perhaps this site is involved in the

response to LPS of 70Z/3 cells and 18-81 subclones A-2 and A-20, but that remains to be determined.

More than one intracellular event may be involved in LPS induction, since 70Z/3 cells respond to stimulation with dextran sulfate by increasing synthesis of μ–heavy chain and expressing it on the cell surface in the absence of light chain (86). By comparison, Gordon et al (87) and Hendershot & Levitt (88) demonstrated that the lymphomas they were studying also express cell-surface μ–heavy chain in the absence of light chain. In addition, one of the A-MuLV transformants derived from cloned pre-B cells—the 4.1 cell line—expresses surface μ despite the fact that no discrete IgL rearrangements can be detected in these cells (89). Further analysis will be required to determine whether or not these $sIgM^+, L^-$ cells represent some previously undefined stage in the differentiative process.

Summary

In a variety of ways transformed B-cell precursors have proven to be an invaluable resource to investigators seeking to define the differentiative pathway by which stem cells mature into immune-competent B lymphocytes. These cell lines have been useful in characterizing the pre-B cell phenotypes in greater detail, with the result that such cells are now easier to identify. Transformed cells also have permitted a detailed analysis of the stepwise process of reorganization of Ig genes by which a functional locus is constructed. Using these cells, researchers can now address questions regarding the regulation of both gene rearrangements and gene expression at the molecular level. Clearly these transformed cell lines will remain powerful tools for the analysis of developmentally regulated processes and ultimately may provide insights into the molecular mechanisms by which eukaryotic cells are immortalized.

REGULATION OF IMMUNE FUNCTION USING Ig-SECRETING TUMOR CELLS

Background

Antigen-specific murine plasmacytomas were the first class of B-cell tumors to be extensively examined with the objective of developing monoclonal B-cell model systems. Significant progress has been made and it is now well established that at least some murine plasmacytomas are responsive to normal immunoregulatory signals (90). Depending on the experimental conditions, various regulatory signals have been shown to influence (*a*) the proliferation and (*b*) the differentiation of plasmacytoma cells; and to alter (*c*) the surface membrane expression and (*d*) the secretion of immunoglobulin. Clearly, distinct signals mediate these effects since each can be

experimentally segregated (91). A sizable literature now suggests that plasmacytoma cell proliferation, differentiation, and immunoglobulin expression can be influenced by processes initiated by antigen-specific, idiotype-specific, or isotype-specific triggering mechanisms. These experimental systems offer exciting possibilities for visualizing the biochemical alterations that occur in a B cell subsequent to receipt of an immunoregulatory signal.

The initial suggestion that murine plasmacytoma cells are susceptible to immunoregulatory signals was provided by the osbservation that mice immunized with a purified myeloma protein were rendered resistant to challenge with otherwise lethal numbers of plasmacytoma cells that expressed the corresponding idiotype. Originally demonstrated by Lynch et al (92) with the TNP-specific BALB/c plasmacytomas MOPC-315 (IgAλ2) and MOPC-460 (IgAκ), comparable findings have been reported by Meinke et al (93), Sugai et al (94), Eisen et al (95), Freedman et al (96), Haughton et al (97), Stevenson et al (98), Krolick et al (99), Perek et al (100), Flood et al (101), Beatty et al (102), Puskas et al (103), and Theilmans et al (104). Miller et al (105) have used an antiidiotype therapeutic strategy in human B-cell lymphoma.

The finding that idiotypic antigens of myeloma proteins could function as tumor-specific transplantation antigens was surprising and appeared somewhat paradoxical. Even though Hannestad et al (106) had shown that murine myeloma cells expressed surface membrane immunoglobulin and proposed that it was a potential target for immune effectors, it nonetheless was surprising that the secreted myeloma protein did not rapidly neutralize the cellular and/or humoral idiotype-specific effectors that mediated the transplantation resistance. Subsequent studies by Rohrer et al (107) demonstrated that the cells of MOPC-315 were cytologically and functionally heterogeneous and that the large, plasmacytoid, M315-secreting cells in the tumor were preferentially lost in the first 24 hr following transplantation into normal recipients. The small, lymphocytoid, nonsecreting, stem cell–enriched tumor population that survived transplantation gave rise, over a period of 10 days, to the original cytologically and functionally heterogeneous tumor. This pattern of cyclic differentiation during in vivo passage has also been reported for hybridoma cells by Tsao & Aldo-Benson (108). Earlier studies by Saunders & Wilder (109), Russell et al (110), and Frondoza et al (111) also provided evidence that murine myeloma cells differentiate as they are passaged in vitro and in vivo. Kubagawa et al (112) have presented evidence that a similar pattern of differentiation occurs in human myeloma clones and have suggested that the extent of clonal involvement may span the pre-B to plasmacyte stages of development.

Following the observation that idiotype-immune mice prevented plas-

macytoma growth, additional evidence for immune regulation of plasmacytomas in vivo came from studies of Paraf et al (113), Barnhill et al (114), Osborne & Katz (115), and Bosma & Bosma (116). In vitro studies by Abbas & Klaus (117), Kim (118), Abbas (119), Bankert et al (120), and Kans et al (121) provided further evidence that antigen-binding plasmacytoma cells could be regulated by antigen-specific mechanisms. Similar conclusions were reached in studies with hybridoma cells by Boyd & Schrader (112), Tsao & Aldo-Benson (108), and Kresina et al (123). In aggregate these studies show that antigen-specific plasmacytoma and hybridoma cells can be influenced by antigen in vivo and in vitro. The nature and intensity of the effects have varied as have the experimental conditions employed in the different systems examined. Many of these interesting studies have been discussed in a review by Abbas (90).

Idiotype-Specific Regulation of MOPC-315

The most extensivly investigated murine plasmacytoma from an immunoregulatory perspective has been the TNP-specific, IgAλ2-producing, BALB/c plasmacytoma MOPC-315. A multiplicity of idiotype-specific immune responses have been identified in BALB/c mice immunized with M315. These have recently been reviewed (124), and only selected aspects will be discussed here. M315-immunized mice inhibit the proliferation of MOPC-315 cells by a mechanism that is idiotype-specific (92) and appears to involve a short-lived, thymus-dependent T cell (125). The effect appears to be cytostatic, not cytotoxic, but further studies are needed to determine the cytostatic mechanism. In addition, M315-immunized mice develop antiidiotypic (Id^{315}) antibodies that are directed to combinatorial determinants (V_{L+H}), some of which are located at or near the TNP-binding site of M315 while others appear to be directed to framework determinants (126). The antiidiotypic antibodies specifically and reversibly modulate surface-membrane expression of M315 in vitro but do not influence M315 secretion or MOPC-315 proliferation (127). The in vivo effects of anti-Id^{315} antibodies are not as yet defined.

M315-immunized mice also develop idiotype-specific suppressor T cells that inhibit secretion of M315 but do not influence MOPC-315 proliferation (128) or the cytologic differentiation of MOPC-315 cells from small lymphocytoid to large plasmacytoid forms. The suppressor T cell is specific for a V_H^{315} idiotope, mediates its effect via a soluble factor, and expresses the surface phenotype Thy-1^+, Lyt1^-2^+ (129). At the effector step it is clear that the suppressor T cells act directly on the actual antibody-secreting cell rather than a secretory-cell precursor. Suppression of M315 secretion is due to a selective inhibition of M315 synthesis by a mechanism that inhibits the expression of λ_2 mRNA in MOPC-315 cells without influencing the

expression of α–heavy chain or J-chain mRNAs (130). It is not yet known whether the λ_2 mRNA inhibition reflects a transcriptional or post-transcriptional level of control.

Although M315 synthesis is blocked in suppressed MOPC-315 cells, normal amounts of the membrane and secreted forms of α–heavy chain mRNA are present, and they function normally in a cell–free translation system (131). These findings have provided strong evidence for a regulatory role of light chain in the expression of heavy chain. It may be that light-chain polypeptides, once synthesized and released from their polysomes, either (*a*) facilitate the release of heavy-chain polypeptides from their polysomes or (*b*) protect heavy chains from degradation. This mechanism could account for the patterns of heavy- and light-chain expression generally observed in B-cell tumors (discussed in 131). A regulatory role of light chain in the full expression of heavy chain appears to operate in the transition of a pre-B cell to an immunocompetent B cell (discussed in 131). A similar mechanism in which a light chain regulates the full expression of a heavy chain may account for the abnormal patterns of expression of human granulocyte adhesion glycoproteins that have been observed in certain families (132).

An interesting aspect of light-chain mRNA regulation in MOPC-315 cells is the coordinate regulation of λ_2 and λ_1 mRNAs (130). In MOPC-315 cells an aberrantly rearranged λ_1 gene produces a truncated mRNA that is translated into a rapidly degraded short λ_1 light-chain polypeptide (133). In suppressed MOPC-315 cells the coordinate regulation of λ_2 and λ_1 mRNAs appears to be mediated by a *trans* mechanism since the aberrantly rearranged λ_1 gene in MOPC-315 cells is located on a different chromosome from the productively rearranged λ_2 gene (134). The considerable nucleotide sequence homology between λ_1– and λ_2–variable regions provides a structural basis for coordinate regulation of the λ family of light chains. Coordinate *trans* regulation of λ_2- and λ_1-gene expression implies the existence of a λ-specific regulatory molecule. It is of interest that Abbas et al (135) observed independent regulation of M11 ($\gamma_{2b}\kappa$) and M315 ($\alpha\lambda_2$) expression in a myeloma-myeloma hybrid line (MPC-11 × MOPC-315) exposed to suppressor T cells specific for the corresponding idiotype. While the molecular details of suppression in those studies were not investigated, the findings are of particular interest since one of the immunoglobulin molecules expressed a κ–light chain, while the other expressed a λ_2–light chain. A light-chain class-specific mechanism could account for independent regulation in a cell producing two immunoglobulins. One predicted consequence of a class-specific inhibition of light-chain expression triggered by idiotype-specific T cells is the suppression of Ig production in that clone independent of heavy-chain class switch. If a

similar mechanism existed for the regulation of κ–light chain expression, then it might be possible to regulate immunoglobulin expression of the entire B-cell repertoire by a mechanism in which an idiotype-specific encounter triggered the expression of either a κ- or λ-regulatory molecule.

The findings of coexpression (133) and coordinate regulation (130) of λ_2 and λ_1 genes in MOPC-315 cells exemplify the penetrating focus that can be achieved in the study of what are usually considered aberrant features expressed in tumor cells.

In addition, the immune responses to idiotypes in the tumor model exhibit an interesting linkage of idiotopic specificity with immune function. Lynch & Milburn (136) have shown that the Id^{315}-specific suppressor T cells that inhibit M315 synthesis and secretion are specific for a V_H^{315} idiotope. In contrast, Jorgensen & Hannestad (137) have shown that M315-immunized BALB/c mice also develop MHC-restricted, Id^{315}-specific helper T cells that are specific for V_L^{315}. These findings are of particular interest because several studies have shown that the Id^{315}-specific antibodies induced in BALB/c mice by immunization with M315 are specific for combinatorial $(V_{L+H})^{315}$ determinants (126). It remains to be seen whether these striking associations and segregations of idiotopes and regulatory functions are fortuitous or have a more fundamental significance.

In a sequence of studies Abbas and colleagues have described an idiotype-specific suppressor system in which M315 secretion is inhibited by splenic T cells from mice immunized intravenously with idiotype-coupled spleen cells (138). Moser et al (139) demonstrated the requirement for an $I\text{-}A^+$ accessory cell in the effector phase of this system.

Antigen-Specific Regulation of MOPC-315

In a sequence of studies Rohrer (reviewed in 90) established that MOPC-315 cells are antigen-sensitive. Using immunization procedures that preferentially induced helper or suppressor T cells specific for sheep erythrocytes, immunized BALB/c mice were implanted with peritoneal diffusion chambers that contained TNP-SRBC, MOPC-315 cells, and accessory cells. Diffusion chambers were sequentially recovered, and MOPC-315 cells assayed for growth, TNP-binding cells, and anti-TNP secretory cells. MOPC-315 growth and differentiation were enhanced in mice with SRBC-specific helper T cells and were suppressed in mice with SRBC-specific suppressor T cells.

The help and suppression were carrier specific. They were observed when MOPC-315 cells were mixed with TNP-SRBC and implanted with SRBC-immunized hosts or mixed with TNP-RRBC (rabbit red blood cells) and implanted into RRBC-immunized hosts. The crisscross experiment did not result in MOPC-315 regulation, i.e. TNP-RRBC did not work in SRBC-

primed mice and TNP-SRBC did not work in RRBC mice. Furthermore, a requirement for linked recognition was observed that had previously been found by Mitchison (140) for normal T:B collaboration. MOPC-315 regulation was not observed in SRBC-primed mice when TNP was present on mouse erythrocytes (TNP-MRBC) with nonhaptenated SRBC also present in the diffusion chamber. In subsequent studies, MOPC-315 regulation was shown to be adoptively transferred to normal mice with T cells, purified through nylon wool, from carrier-primed donors; adoptive transfer was abrogated by treatment of the donor cells with anti-Thy-1 serum plus complement (141). Carrier-specific regulation of MOPC-315 requires histocompatible macrophages in the diffusion chamber with the MOPC-315 cells and the TNP-carrier. As with idiotype-specific regulation of MOPC-315, distinct sets of carrier-specific T cells regulate clonal proliferation and secretory differentiation. The regulatory capacity of this system is considerable, as is demonstrated by studies in mice with advanced, disseminated MOPC-315 tumors (142).

In an interesting sequence of studies (reviewed in 90), Abbas demonstrated that DNP-conjugated IgG could inhibit synthesis and secretion of M315 by MOPC-315 cells in vitro without influencing MOPC-315 growth. DNP-Ig was rapidly endocytosed by MOPC-315 cells, and electron microscopic studies showed abundant localization near the perinuclear Golgi zone. Detailed biochemical studies were not carried out, but this system may be a useful model for investigation of suppression by tolerogenic antigens and antigen-antibody complexes.

Isotype-Specific Regulation of MOPC-315

While most studies of B-cell regulation that have used a tumor model have employed the tumor cells as monoclonal *targets* of immunoregulatory signals, a number of studies have established that malignant B cells can also be *inducers* of immunoregulatory signals. Examples include the plasmacytoma-induced macrophage activation that leads to immune deficiency (143) and the osteoclast activation that results in skeletal demineralization (144). If these two effects are "exaggerated" but otherwise normal functions of Ig-secreting cells, it remains to be determined how these activities contribute to immune homeostasis.

A striking example of immunoregulatory induction by B-cell tumors is the pattern of T-cell alteration seen in murine and human myeloma (145). These B-cell neoplasms are accompanied by the development of large numbers of regulatory T lymphocytes that express surface receptors specific for the heavy-chain class of the corresponding myeloma protein. T_α cells are increased with IgA-secreting tumors (146), T_Σ cells with IgE (147), T_γ cells with IgG, and T_μ cells with IgM (148). The FcR^+ T cells that increase in

mice with plasmacytomas and hybridomas are Thy-1$^+$, L3T4$^-$, Lyt1$^-$, Lyt2$^+$, adult-thymectomy-sensitive lymphocytes (149). The T_α cells that develop in mice with MOPC-315 tumors are IgA-specific suppressor T cells that can inhibit normal IgA responses (150) and can suppress proliferation and IgA secretion by MOPC-315 cells (151). The phenotypic and functional properties of the FcR$^+$ T cells in myeloma suggest that the cells reflect exaggerated responses of isotype-specific regulatory circuits. B-cell tumors that exhibit such regulatory functions may provide powerful models for investigating the mechanisms that control isotype selection and expression in B cells.

Summary

MOPC-315 is an extensively characterized tumor that is both a *target* and an *inducer* of immunoregulatory signals. A single effect, e.g. inhibition of M315 secretion, can be triggered by separate idiotype-, antigen, and isotype-specific mechanisms. An issue of considerable interest is whether different regulators trigger the same final common pathway. For example, do the antigen-specific and isotype-specific suppressor mechanisms that inhibit M315 secretion do so by regulation of λ_2-mRNA expression as has been shown for idiotype-specific suppression?

TUMOR MODELS OF B-LYMPHOCYTE FUNCTION AND REGULATION

The I.29 Lymphoma Model of Heavy-Chain Class Switch

I.29 is a monoclonal B lymphoma of I/St mice (152). When grown in vivo this tumor consists of cells expressing IgM and IgA on their surfaces. Both isotypes express the same idiotype (153) and are encoded by the same V_H gene (154). In an elegant study Stavnezer et al (155) have developed this tumor system as a model for studying heavy-chain class switching. Clones of IgM$^+$ I.29 cells have been induced by LPS to switch to IgA, IgE, or IgG2a. Fifteen days after exposure to LPS, up to 90% of the originally IgM$^+$, IgA$^-$ I.29 cells become IgM$^-$, IgA$^+$. In actively switching cultures up to 50% of the cells simultaneously express IgM and IgA, indicating that the appearance of IgA is due to isotype switching and not to clonal outgrowth.

Prior to switching, the I.29 cells contain two H chromosomes bearing two rearranged μ genes—an expressed μ gene (which has undergone VDJ_4 recombination) and a nonexpressed μ gene that has undergone a DJ_2 recombination. In the IgM$^+$ cells the C_H genes 3′ to the μ genes are present in the germline configuration on both chromosomes. Southern blots of Ig

heavy-chain genes in I.29 cells before and after switching show that isotype switching is accompanied by DNA recombinations that occur within, or immediately 5′ to, the switch sequences. Cells that have switched to IgA expression contain rearranged, expressed α genes, produced by recombination between the S_μ region within the expressed μ gene and the Sα region (155).

The I.29 tumor is an attractive model for investigating molecular mechanisms involved in heavy-chain class switching. It has the major advantage that the switch can be induced by LPS, one of several features the I.29 model shares with normal B cells, and antiidiotypic antibody synergizes the LPS induction (155). Isotype switching has been investigated with other tumor systems such as pre-B-cell lymphomas (32) and a number of myelomas and hybridomas (156), but in these systems the switch is: (*a*) spontaneous, not induced; (*b*) an extremely low frequency event; (*c*) not accompanied by DNA recombinations in the switch sequences; and (*d*) often a switch to the C_H gene located immediately 3′ to the gene being expressed. The I.29 tumor, therefore, provides a clonal model of isotype-switching that appears to be physiologically relevant. It may provide a powerful experimental system for addressing issues such as: (*a*) the linkage of membrane receptors to the activation of specific switch recombinases; (*b*) the role of T cells and T-cell factors in the switch from IgM to IgA and IgE; and (*c*) the nature and significance of switch recombinations that occur in the allelic, nonexpressed heavy-chain genes.

In addition to isotype switching, I.29 cells may be useful models to investigate the transition of B lymphocytes to plasmacytes. Sitia et al (153) have reported that LPS induces $sIgA^+$ I.29 cells to increase in diameter, to become $sIgA^-$, to develop intense cytoplasmic IgA, and to increase IgA secretion 50-fold. Induction of high-rate secretion was also observed by Hamano & Asofsky (157) when $mIgM^+$ murine B-hybridoma cells were exposed to anti-IgM antibody. Interestingly, a subline of this hybridoma produced IgG2a as well as IgM when exposed to anti-IgM antibody.

Regulation of Growth and Differentiation in B-Cell Lymphomas and Leukemias

There are now many examples of B-lymphocyte tumors that differentiate spontaneously or following exposure to a variety of immunoregulatory signals. In humans the studies of Fu et al (158) showed that certain clones of chronic lymphocytic leukemias (CLL) contained cells at different stages of differentiation from $mIgM^+$, $mIgD^+$ lymphocytes to $mIgM^{+/-}$, $mIgD^-$, IgM-secreting plasma cells. They showed that CLL cells could be induced to differentiate by allogeneic helper-T-cell factors or by pokeweed-mitogen

stimulation in the presence of autologous T cells. Differentiation was not dependent on cell division and could be blocked by anti-IgM or anti-idiotypic antibodies.

An intense research effort has been directed towards identification and characterization of T cell–derived lymphokines that affect B-cell growth and differentiation, to develop an understanding of their mechanisms of action. Vitetta, Uhr, and colleagues have employed BCL_1, a BALB/c B-cell prolymphocytic leukemia/lymphoma (159), in some of these studies Brooks et al (160) have established in vitro clones of BCL_1 that express high surface IgM, low surface IgD, and variable levels of surface Ia. In the presence of $BCDF_\mu$, the BCL_1 cells exhibit a 10–90-fold increase in IgM secretion and comparable increases in cytoplasmic IgM. Northern blotting using a μ-specific DNA probe showed that prior to the addition of $BCDF_\mu$ a 1.7 kb mRNA for membrane μ chain was the predominant species of cytoplasmic μ-specific RNA in the BCL_1 cells. After exposure to $BCDF_\mu$ and induction of IgM secretion, there was a marked increase in the quantity of the 2.4 kb mRNA encoding the secreted form of the μ chain. The BCL_1 system has been a productive model for investigating B-lymphocyte growth and differentiation.

There is now a sizable literature describing studies in which B-lymphocyte tumors have been used to investigate a feature known to be expressed by normal B lymphocytes. Berman et al (161) used this approach to examine the structure of membrane IgM. Paige et al (31) and Boyd et al (162) studied the effect of LPS on WEHI-231, a B-cell lymphoma that expressed surface IgM. They found that LPS induced a marked reduction in surface IgM (7S) and a concomitant increase in secretion of (19S) IgM.

The basis for inactivation of normal B cells by antiimmunoglobulin antibodies was investigated by Boyd & Schrader using WEHI-231 (163). They showed that low concentrations (0.1 μg/ml) of anti-IgM inhibited WEHI-231 proliferation and resulted in cell death. This finding suggested that WEHI-231 might be a tumor model of anti-IgM-induced B-cell tolerance (164). McCormack et al (165) showed that WEHI-231 cells exposed to anti-IgM undergo a single round of division but that further proliferation is inhibited. Interestingly, this arrest of growth by a specific receptor-mediated signal is preceded by a 25–50-fold decrease in the cytoplasmic levels of *c-myc* mRNA. The drop in *c-myc* mRNA is highly selective in that other mRNAs are not affected. Further studies are needed to determine the molecular basis for the dramatic *c-myc* mRNA decrease and to identify the mechanism that leads to death of WEHI-231 cells following exposure to anti-IgM antibodies. While perturbations of the *c-myc* locus and dysregulation of *c-myc* expression have been identified in many human, murine, and avian B-cell tumors, WEHI-231 is an example

of a B-cell tumor in which the germ-line *c-myc* proto-oncogene is not rearranged. Furthermore, *c-myc* appears to be under strict positive and negative regulatory control in WEHI-231 cells by a mechanism that can be influenced by engagement of surface-membrane IgM. This tumor appears to provide a useful model for investigating the mechanisms of B-cell inactivation by antiimmunoglobulin.

Tumor Models for the Study of Immunoglobulin Secretion

In an interesting series of experiments Hickman and colleagues have used murine plasmacytoma cells to investigate some of the biochemical requirements for immunoglobulin secretion. They observed that tunicamycin blocked the secretion of IgA and IgE by inhibiting heavy-chain glycosylation (166). Interestingly, nonglycosylated M315 could be reexpressed at the surface membrane of tunicamycin-treated MOPC-315 cells (167). Additional insight into the biochemistry of IgA secretion has come from studies of 315/P, a nonsecreting variant of MOPC-315 that expresses surface-membrane M315 (168). This clone expresses several other features characteristic of presecretory B cells (169). Compared to wild-type M315 cells, this variant exhibits a marked elevation in sugar-nucleotide pyrophosphatase which essentially depletes the pool of glucosylated nucleotides that are required for heavy-chain glycosylation (170). Glucocorticoids induce high levels of sugar-nucleotide pyrophosphatase activity in the M315-secreting wild-type MOPC-315 cells, and this results in inhibition of M315 secretion (171). These studies have identified potentially important biochemical control points for the regulation of secretion. They are particularly interesting in view of the effect of glycosylation on regulatory functions that have been ascribed to some T cells that influence IgE secretion (reviewed in 172).

SUMMARY AND CONCLUSIONS

B-cell tumors have been extraordinary sources of information about antibodies, their genes, and the cells that express them. An important principle that has emerged from the study of lymphoid tumors is that the long-held view that malignant lymphoid cells are "frozen" at a fixed point in differentiation is not generally valid. Presentation of immunoregulatory signals to transformed B cells can profoundly influence their proliferation, morphology, differentiation, gene expression, and immunoglobulin synthesis. In addition to their responsiveness to immunoregulatory signals, some tumors of B lineage elaborate immunoregulatory signals. Until quite recently B-cell tumors were used primarily as monoclonal sources of molecules of immunological interest. While they continue to be important

sources of receptors, growth and differentiation factors, differentiation antigens, and immunoregulatory factors, they are being used with increasing frequency to define the molecular events that occur in B cells subsequent to receipt of an immunoregulatory signal. While the use of tumor cells as models of normal cells is often viewed with some skepticism, it is difficult to find examples wherein tumors have been misleading. Quite to the contrary B-cell tumors have regularly provided powerful tools for dissecting the molecular events that underlie B-cell development, function, and regulation

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CONTENTS

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MECHANISM OF CYTOTOXICITY BY NATURAL KILLER (NK) CELLS[1]

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INTRODUCTION

Natural killer (NK) cells were discovered about 13 years ago during studies of cell-mediated cytotoxicity against tumor cells (see 1 for review). Although investigators expected to find specific cytotoxic activity in tumor-bearing individuals against autologous tumor cells or against allogeneic tumors of similar or the same histologic type, appreciable cytotoxic activity was evident when lymphocytes from normal individuals were observed. It became clear that such cytotoxic activity was mediated by a subpopulation of effector cells that have come to be known as NK cells. The characteristics of NK cells and their possible relationship to T cells or macrophages have been extensively studied, and a previous review in this series has summarized much of the available information (2). One of the most consistent features of NK cells, which has permitted their purification and detailed comparison with other types of effector cells, is their close association with a morphological subpopulation of cells, the large granular lymphocytes (LGL) (3).

In addition to extensive efforts to characterize NK cells and to determine their in vivo relevance, particularly in regard to their possible role in

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immune surveillance and in resistance to progressive growth and metastases of tumor (4), there has been much interest in elucidating the mechanism of cytotoxicity by NK cells (5). Recent progress makes it now possible to assimilate most of the available evidence in a model that divides the interactions between NK cells and NK-susceptible target cells into several phases (Figure 1).

Clearly the NK cell first has to bind to the target cell through a receptor that recognizes structures on the target cells. The second phase involves the triggering or activation of intracellular processes in the NK cells, probably

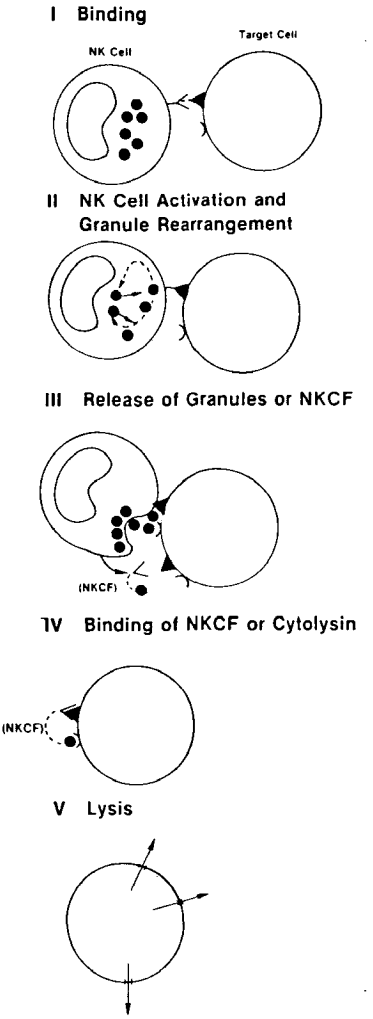

Figure 1 Model for phases in interaction between NK cells and NK-susceptible target cells.

affecting the cytoplasmic granules and leading to the rearrangement of the granules and other cytoplasmic organelles towards the site of binding with the target cell. Demonstration of potent and rapid cytotoxic activity by isolated LGL granules makes it likely that the next step is release of some granules or their contents from the effector cell at, or in close proximity to, the junction with the target. Then molecules of the granule cytolysin would bind to the target cell, presumably at sites separate from those recognized by the NK receptor. One might expect that most of the target selectivity for lysis is based on the NK receptors, and the finding that LGL granules have less specificity than NK cells is consistent with this expectation. The soluble NK cytotoxicity factor (NKCF) might be an alternative but less efficient mechanism of lysis or it might be related to the granule cytolysin. There is evidence for a binding step in the interaction of NKCF with target cells, since NKCF can be absorbed out by susceptible targets and these then lyse without any further exogenous exposure to granules or NKCF.

The final step in the pathway of cytotoxicity is the lysis of the target cells. The details of the lytic event remain to be elucidated. An intriguing possibility is that the cytolysin causes a channel or ring-like hole to be introduced into the target structure. Alternatively, the cytolysin may make a channel which then allows a toxin to enter the target cell and kill it.

Less information exists about how NKCF mediates lytic effects and whether that process is similar or distinct from that associated with LGL granule cytolysin.

A recent opportunity to explore the nature of NKCF and other possible soluble mediators has come from the availability of several highly purified cytotoxic molecules, e.g. lymphotoxin (LT) and tumor necrosis factor (TNF) and antibodies against these factors. Studies have been initiated to determine the possible relationship of NKCF to these well-defined factors and to evaluate the role of such molecules in NK activity.

Clearly, this model is an oversimplification of a complex sequence of events, and Targen et al (6) have carefully dissected out several distinct phases, which are subsequent to the binding of NK cells to target cells. However, this model does provide a framework for classifying the mechanism of action of the many factors demonstrated over the last several years either to increase or to inhibit NK activity. It is now possible to arrange most of these factors in a sequence according to whether they are affecting binding or one of the later events.

The present review summarizes much of the available information on each of the phases of the NK cell–target cell interaction that can lead to the lysis of target cells. The review particularly emphasizes the nature of the recognition receptors on NK cells, the complementary target structures that lead to conjugate formation, and the cytotoxic molecules that mediate lytic activity.

PHASES IN THE CYTOLYTIC PROCESS

Binding of NK Cells to Target Cells

NATURE OF TARGET-CELL STRUCTURE As indicated in the previous section, the cell-mediated cytolytic process can be divided into a number of distinct steps. The initial step in cytotoxicity by all types of cytotoxic lymphoid cells, including natural killer (NK) cells, involves binding of the effector cell to the target cell. For cytolytic T cells, much is known about the interacting structures on the surface of both the effector cells and the target cells responsible for this interaction. The T-cell receptor and its associated structures have been well defined (7), and it is clear that recognition of the major histocompatibility complex (MHC) in association with specific antigen on the target cell is usually required for binding and subsequent lysis (8). In contrast, little information exists as yet about the recognition structure on the surface of NK cells or the target-cell structure(s) they recognize. We review here the evidence obtained thus far.

While it is clear that a broad array of tumor target cells are lysed by NK cells, there is nonetheless a selective pattern of killing, with some lines distinctly resistant to NK activity (9, 10). The susceptibility of such a wide spectrum of target cells to recognition and lysis by NK cells could be due to expression of a common determinant. Alternatively, multiple subsets of NK cells may each recognize a different determinant. From studies using adsorption procedures (11, 12) or cloning of effector cells (13–15), the evidence favors the existence of at least several subsets of NK cells, some with broad reactivity and others with narrower patterns of cytotoxic effects.

Insight into the nature of the NK target structure and its heterogeneity would be much increased by determination of the biochemical nature of the target cell determinant. However, to date very few studies have directly addressed this question. The paucity of biochemical studies on the NK target structure can be attributed (*a*) to limitations, at least until recently, in the methodology to isolate and purify large numbers of NK cells, and (*b*) to unavailability of a rapid quantitative method to assess the interaction of NK cells with soluble membrane-derived materials.

A number of indirect approaches have been used to investigate NK target structure(s). Treatment of targets with various enzymes (e.g. proteases, lipases) and agents like tunicamycin have led to the suggestion of a role for a glycoprotein or glycolipid structure (16). A second, more indirect, approach used specific reagents such as antitransferrin receptor (17–20), antilaminin (20a), or antitarget antibodies (21). However, none of these studies yielded information that accounts for NK activity against an array of susceptible target cells; of more concern—most such inhibitory agents block NK activity at step(s) subsequent to binding of the effector

cells to target cells. However, digestion of target cells with proteases can result in the loss of recognition by NK cells (22), implying that surface proteins are a part of the target structure that is recognized.

A more direct approach would be to isolate membrane components from NK-susceptible targets and to measure their effects on the binding of NK cells to targets. Roder et al (23, 27) reported that high molecular weight (140–200 kd) glycoproteins can inhibit conjugate formation of normal mouse spleen cells with NK-susceptible targets, but a more detailed characterization of the target-cell molecules and the determinants recognized by mouse NK cells has not been reported. The ability to isolate highly purified NK cells, using discontinuous Percoll gradients, prompted a biochemical characterization of NK target-cell molecules involved in binding of human NK cells to the highly NK-susceptible target K562 (24). The use of highly purified effector cells was important since the previous study by Roder et al (23) used unfractionated mouse spleen cells, which have been shown to contain a high percentage of cells that bind to NK-susceptible targets but that lack any detectable cytolytic activity (25). A detection system using highly purified NK cells avoids inhibition by non-NK cell binders, since the majority (>80%) of binders have lytic activity against the target cells (26). Solubilized membrane proteins from K562 target cells were purified by various chromatographic procedures, reconstituted with exogenous lipid, and then tested for their ability to inhibit conjugates of LGL and target cells (24). The evidence that the inhibitory material was the target-cell molecule(s) recognized by NK cells was provided by the specificity of the inhibition. Membrane material from the human NK-susceptible target K562 inhibited conjugate formation between human LGL and several human NK-susceptible target cells but did not affect binding between human LGL and antibody-coated targets nor between rat NK cells and their NK-susceptible mouse or rat targets. The characteristics of the conjugate-inhibitory materials are summarized in Table 1. Results from the studies with human target cells indicate that the target-cell structures are protein in nature (27). The inhibitory material was sensitive to trypsin and lost activity after incubation at 65° for 40 min, but not after incubation at 56°. The material could be purified by a variety of lectins with strong binding to Con A and weak binding to peanut agglutinin. The size of the inhibitory molecules appeared to cover a broad range of molecular weights, with the range in the human from 30–165 kd and in the mouse, 140–240 kd. Collectively these studies indicate that inhibition of binding of effector cells to target cells by materials that have been separated by standard biochemical methods can be used to elucidate the structure recognized by NK cells. The available evidence regarding the detailed characteristics of the molecule(s) remains limited in regard to (*a*) the

Table 1 Nature of isolated NK target structures

Characteristic	Species	Nature	Reference
Composition	Human	Protein	26
	Mouse	Protein	27
Sensitivity to inactivation	Human	Trypsin-sensitive	24
	Human	Heat-labile (65° for 40 min)	24
Adsorption to lectins	Human	Con-A (strong)	24
	Human	Ulex europa (weak)	24
	Human	Peanut [Arachis] (weak)	24
Molecular weight range of inhibitory material	Human	Broad (30–165 kd)	24
	Mouse	Broad (140–240 kd)	23

relationship of binding structures from one target to the next, (*b*) what regulates the expression of these target cell structures, and (*c*) whether they are associated with viral or oncogene products. Current data do not indicate the nature of the determinant(s) recognized by NK cells; they only indicate the characteristics of molecules bearing these determinants. It is possible that the determinants recognized by NK cells may be protein and/or carbohydrate in nature. That treatment with trypsin destroyed the inhibitory activity does not exclude a carbohydrate determinant since the protein may be required for efficient interaction of the soluble inhibitory material with lipid. Evidence for such a requirement exists for the human T-cell receptor that has a transmembrane lipophilic sequence (28, 29). Likewise the heat inactivation of the target structure may be attributable to a requirement for tertiary protein conformation for its reconstitution into lipid vesicles or for presentation to the effector cell; it does not necessarily imply a protein determinant. Studies to be discussed later indicate that initial binding is only one step in these cytolytic mechanisms. A number of systems have been defined in which inhibition of lysis is due to postbinding events. Also, some mutant NK-resistant target cells are not deficient in their binding to NK cells, but rather in their ability to activate NK-cell cytolytic mechanisms (30, 31), indicating that some postbinding activation structures or signals are not recognized by the effector cell. Therefore, a target cell may be easily recognized by the effector cells but may be unable to activate the lytic machinery or, alternatively, may possess molecules that interfere with subsequent events required to lyse the target cell.

THE RECOGNITION RECEPTOR ON NK CELLS Although the binding of NK cells to target cells is clearly required to activate the lytic process of these cells, the mechanism(s) by which these effectors recognize target cells is still not clear. Most of the data regarding the NK-recognition receptor only

serve to show what it is not. For instance, although NK and antibody-dependent cellular cytotoxic (ADCC) activities may be mediated by the same effector cell, blocking of the receptor for the Fc portion of IgG (FcγR) inhibits only ADCC activity and has little or no effect on NK activity (32). This demonstrates that the NK receptor is not simply the FcγR binding to cell-bound antibody on the target. Similarly, the NK-recognition receptor is unlikely to be immunoglobulin (Ig) since Ig-like molecules are absent from the surface membrane of LGL (33, 34), and treatment of lymphocytes with anti-Ig and complement has no effect on their NK activity (35).

It has been suggested that the recognition of murine NK-susceptible target cells by NK cells is via a laminin-like structure (20a), but use of purified populations of human LGL has so far not obtained similar results (J. Ortaldo, unpublished observations). Therefore, the role of laminin and/or laminin receptors in target-cell recognition by LGL is still unclear.

It has also been suggested that murine NK cells and cytotoxic T lymphocyte (CTL) and other clones with NK-like activity express mRNA as well as rearrangements of the genes coding for the β-chain of the T-cell receptor (36). Experiments in the human cells using $T3^+$, but not $T3^-$, lymphocyte clones with NK-like activity have produced similar data (37). The authors suggest that the T-cell receptor on such cells acts as the recognition receptor for NK-like activity, since antibodies to the idiotypic determinants of the T-cell receptor blocked cytotoxic activity against K562 (38). However, even in those studies, some clones with NK-like activity had no detectable expression of T-cell receptors, indicating that other structures can recognize NK-susceptible targets. In addition, data from our laboratory indicate that the NK-recognition receptor is different from the T-cell receptor. First, in the rat, LGL tumor lines with high NK activity have no genomic rearrangement of the T-cell receptor β-chain and no detectable complete 1.3-kb mRNA for this structure (39). Second, freshly isolated, highly purified populations of rat, mouse, and human LGL have no detectable 1.3-kb mRNA or β-chain gene rearrangement, in spite of very high NK activity (40). Third, the addition of anti-T3 antibody, which recognizes a portion of the human T-cell receptor complex, has no effect on the NK activity of freshly isolated human LGL or $T3^+$ cytotoxic clones with NK-like activity (J. Ortaldo, unpublished observations). Finally, Binz, Wigzell, and associates (41) have reported that addition of antiidiotype antibodies to CTL lines with both antigen-specific and NK-like activity inhibited only the antigen-specific cytotoxicity but did not affect the NK-like activity of these clones.

The likely reason for divergent results regarding fresh mouse NK cells (36, 40) is related to the difficulty in obtaining highly purified NK cells without appreciable contamination by T cells (i.e. $< 5\%$ T cells) from mouse

spleen, and the previous positive report (36) is probably due to insufficient purification of the effector cell population. The detection of T cell–receptor gene rearrangement and/or expression in in vitro cultured cells may be explained by the appearance of T cell–receptor molecules as well as T3 in some NK cells when cultured (42) or by the development of NK-like activity in typical T cells when cultured in IL-2 (43). In either case, the expression of the T-cell receptor is unlikely to be related to the expression of NK-like receptors on these cells.

Taken together, these results indicate that the NK-cell recognition receptor is not identical to the previously described T-cell receptor complex and its molecular nature remains elusive. Perhaps use of a molecular biological approach similar to that successfully used for the T-cell receptor will lead to the elucidation of the structure(s) by which NK cells recognize target cells.

Activation of NK Cells and Rearrangement of Granules

After conjugate formation, the next interaction appears to involve the triggering or activation of intracellular processes in the NK cells, probably affecting the cytoplasmic granules and leading to the rearrangement of the granules and other cytoplasmic organelles towards the site of binding with the target cell. Several years ago, Saksela et al (44) presented evidence for such rearrangement after binding to target cells, and recently Dennert et al (45) have obtained similar evidence. Based on the isolation of the LGL granules and the demonstration of their potent and rapid cytotoxic activity (see section on LGL Granule Cytolysin), it is likely that the next stage involves the release of granules close to the junction with the target.

Release of Cytotoxic Molecules

Recently, an appreciable amount of experimental evidence has accumulated for a mechanism of NK activity involving secretion of cytolytic molecules. This evidence includes (*a*) rearrangement of cytoplasmic organelles and release of granules from NK cells following their binding to target cells (46, 47); (*b*) decreased NK activity in Chediak-Higashi patients (48–50) and Beige (bg/bg) mice (51) that bear mutations leading to abnormal formation of lysosomal granules; (*c*) the reported inability of agranular lymphocytes to kill after they contact tumor cells (52); (*d*) a reduction in NK activity by strontium (53, 54), which promotes leukocyte degranulation (55); (*e*) a requirement for lipid metabolism (transmethylation and phospholipase A_2 activity) for both secretion of lysosomal enzymes and NK-cell activity (56, 57); and (*f*) the inhibition of NK activity by lysosomotropic amines that interfere with lysosomal function (58). Demonstrations that NK cells release soluble cytolytic factors (NKCF)

upon incubation with NK-susceptible target cells or lectins (59, 60) provide further evidence that a secretory process is involved.

LGL GRANULE CYTOLYSIN To evaluate directly the role of LGL granules in the lysis of tumor cells, the cytoplasmic granules from rat LGL tumors have been purified (61, 62). These cells provide a convenient and uniform source of highly active cytolytic cells with NK specificity (63, 64). The granules contain a potent, calcium-dependent cytolytic material, termed LGL granule cytolysin, not present in the cytoplasmic granules of other noncytolytic leukocytes (62). The characteristics of the cytolysin are summarized in Table 2. LGL-cytolysin is a protein of approximately 60 kd that lyses a wide range of target cells in a rapid and Ca^{2+}-dependent manner. When tested on liposomes, LGL-cytolysin induced a rapid release of internalized carboxyfluorescein (62). As previously seen on lysed NK or ADCC targets (46, 65, 66), characteristic ring-like structures appeared, inserted into the lipid membrane. Penetration of negative stain into the liposomes correlated well with the presence of these pore structures. Together these data provide strong support for the model that assumes LGL granule-derived pore insertion into the lipid layer as a mechanism of cytolysin activity.

The activity of LGL-cytolysin clearly supports the model for lymphocyte cytotoxicity involving granule exocytosis after target-cell recognition. However, it is not easy to design definitive experiments to test whether granule cytolysins are responsible for the lethal damage inflicted by cytotoxic lymphocytes. One approach is to use rabbit antibodies against the purified LGL tumor granules. Fluorescence microscopy shows that such rabbit antibodies stain cytoplasmic granules in LGL tumor cells, LGL, and CTL, but not in normal splenocytes, thymocytes, or peripheral T cells (67). The antibodies do not stain the plasma membranes of LGL

Table 2 General characteristics of LGL granule cytolysin

Characteristic	Attribute
Lymphocyte source	Fresh LGL, rat LGL tumors
Kinetics (37°C)	Rapid (1–2 min)
Divalent cation requirement	Ca^{+2} or Sr^{+2}
Sensitivity of targets to lysis	SRBC > nucleated cells > LGL
Molecular weight	60–62 kd
Stability	Heat-labile, pronase-sensitive, inactivated following addition of Ca^{+2}
Concentration required for lytic activity	$>4 \times 10^{-11}$ M (3 ng/ml)

and granule staining can be detected only after permeabilization of the membrane. By Western blots, antigranule antibodies react with four of the five major granule proteins (67), and IgG from the antigranule sera specifically block granule cytolysin activity (67). Importantly, $F(ab')_2$ fragments of these antibodies specifically block the lytic activity of purified rat LGL in NK and ADCC assays in addition to the cytolysin activity (67) (Table 3). The antibodies do not interfere with binding of LGL to target cells, an expected result for antibodies to cytoplasmic granules. We regard these experiments with antigranule antibodies as strong direct evidence for a role of granule components in NK activity. Presumably the antibodies have access to the granule cytolysin upon its release from the NK cells and prior to its effective interaction with target cells bound to the effector cells.

As summarized in Table 2, the LGL granule cytolysin has much broader specificity for target cells than that seen with NK cells. NK-resistant tumor cell lines and even LGL themselves are lysed by the cytolysin, and sheep erythrocytes—not susceptible to NK activity—are particularly sensitive indicators of cytolysin activity (62). This major difference in specificity of NK cells and cytolysin, and the lack of lysis of NK-resistant third-party cells during an NK assay, need to be explained to postulate a central role for LGL granule cytolysin in the lytic process of NK cells. It is likely that during the interaction between NK cells and targets, the granule cytolysin is released mainly in the small intercellular space at the point of conjugate formation. Extracellular Ca^{2+} should rapidly inactivate the cytolysin that escapes into the surrounding medium before it reaches other target cells. Thus, the specificity of the NK reaction would be defined mainly by the initial recognition of susceptible targets, with cytolysin released only upon

Table 3 Effect of antigranule Ab on cytolysin and on NK and ADCC activity

Treatment	^{51}Cr-release[a] (%)			Conjugates of LGL[b] (%)	
	SRBC	YAC-1	P815+Ab	YAC-1	P815+Ab
None	NT[c]	34±1	38±3	18±5	34±1
PBS control	78±2	34±3	37±3	20±2	33±2
Anti-TNP control[d]	81±3	30±6	38±2	17±4	26±1
Anti-LGL granule[d]	1±1	3±1	14±1	18±5	35±2

[a] With either 10 units cytolysin (versus SRBC targets) or purified rat LGL (versus YAC-1 or P815+Ab), effector/target = 25:1.
[b] Effector/target = 2:1 at 37°C for 5 min.
[c] Not tested.
[d] $F(ab')_2$ fragments of rabbit antibodies at 300 μg/ml in the assay.

an effective interaction. According to this model, it would not be necessary to have specificity at the lytic molecule phase as well.

NK CYTOTOXIC FACTOR (NKCF) Wright & Bonavida described a soluble factor that selectively lyses NK-susceptible target cells (59, 60, 68). This factor, produced during the interaction of mouse spleen cells with NK-susceptible targets, was termed natural killer cytotoxic factor (NKCF). Similarly, human NKCF is released upon incubation of human peripheral blood lymphocytes (16, 69) or of purified large granular lymphocytes (16, 69, 70) with NK-susceptible targets or mitogens. Table 4 summarizes some of the characteristics of NKCF.

Both human and murine NKCF appear to be protein in nature, in that they are inactivated by trypsin treatment (72). Sialic acid does not seem to be an important part of the molecule since activity is maintained after treatment with neuraminidase (72). NKCF is susceptible to sodium periodate and other reducing agents (72), suggesting the involvement of carbohydrate and/or S-S bonds in the functional portion of the molecules. NKCF seems relatively unstable compared to other secreted proteins in that stability at 4°C or in the frozen state is limited to days or weeks (16, 68, 69). On molecular sieving columns both human and mouse NKCF show one peak of activity at 20 kd and another at 40 kd (16, 72).

Table 4 Characteristics of NKCF

Properties	Nature	Reference
General		
Cellular source	LGL	16, 69
Stimuli for release	NK-susceptable targets or mitogens	16, 68, 71
Rate of release increased by interferon	Release detectable in 3–6 hours	16, 70
Kinetics of lysis	Slow (>10 hour) required for detectable activity	16, 68, 69
Pattern of target cell susceptibility	Similar to NK cells	16, 72
Biochemical		
Molecular weight	20–40 kd	16, 72
Stability at		
−20°C	Weeks to months	16, 68, 69
4°C	Days to weeks	16, 68, 69
56°C	Hours	16, 68, 69
>65°C	<10 minutes	16, 68, 69
Inactivation by enzymes	Sensitive to trypsin	16, 72
	Insensitive to neuraminidase	72

Several observations suggest that NKCF is involved in the lysis by NK cells: (*a*) NKCF can be produced by highly purified LGL (69, 70) that account for virtually all NK activity of peripheral blood lymphocytes (73); (*b*) the ability of various target cells to stimulate release of NKCF correlates well with susceptibility to lysis by NK cells (16, 68, 71); (*c*) the specificity of the effects of NKCF is similar to that of NK activity; NKCF has lytic activity mainly against NK-susceptible target cells (16, 72); and (*d*) the amount of NKCF activity released from effector cells can be substantially augmented by pretreatment with interferon (16, 70), paralleling the ability of this cytokine to boost NK activity.

In contrast to these similarities between the characteristics of NKCF and NK activity, the kinetics of lysis are quite different. The lytic activity of NK cells can be detected within 20 min and reaches close to maximal levels by 3–4 hr against highly susceptible target cells, whereas the activity of NKCF usually cannot be seen before 6 hr and maximal levels of lysis usually require incubation periods of >18 hr (16, 68, 69). It is possible to reconcile these differences and retain consideration of NKCF as an important mediator of NK activity. One might postulate that in the interaction between NK cells and susceptible targets, NKCF is secreted at a high concentration into the tight junction between the cells where it is considerably more efficient and protected from inhibition by proteins in the surrounding medium. In contrast, the concentration of NKCF in the culture medium would be expected to be very much lower and directly susceptible to rapid inhibition by the serum proteins in the medium. Such features would also account for the lack of lysis of unconjugated, bystander cells during the NK assay. Recent studies using partially purified and concentrated NKCF have resulted in accelerated kinetics of lysis (16, 69). This result is consistent with the hypothesis that the rate of lysis is proportional to the concentration at the intercellular junction.

NK inhibitors have provided an opportunity to dissect the step(s) in the lytic process. Agents known to inhibit NK activity at a post-binding phase have been tested for their ability to block either (*a*) the production and/or release of NKCF when present during the 24-hr period of interaction between the effector cells and target cells, (*b*) binding of NKCF to target cells, when present during the 6-hr period required for maximal adsorption of NKCF (16), or (*c*) during the terminal lytic phase when the agent is added to target cells precoated with NKCF. Table 5 summarizes the results. The majority of the agents tested—those that remove divalent cations, ATP or cyclic AMP, prostaglandins, ammonium chloride, strontium chloride, and monensin (an inhibitor of microfilament and microtubule movement)—were potent inhibitors of the production and/or release of NKCF. Most of these had no effect on the subsequent binding of NKCF to targets or on the

lytic stage itself. The ability of antiserum to isolated cytotoxic granules from LGL tumors (see section on LGL Granule Cytolysin) to inhibit production or release of NKCF was not analyzable because the antibody could not be removed from the culture medium and because it inhibited the subsequent binding of NKCF to target cells. The other agents were easily removed from the reaction mixture by dialysis and, therefore, could be assessed for their effects on factor production or release. That the majority of agents that inhibited NK activity inhibited production and/or release of NKCF supports the concept that these NKCF-related processes are key steps in NK activity. These observations also indicate that binding is not sufficient to activate NK cells and that postbinding events are necessary for initiation of the lytic process. This hypothesis has been substantiated further by Wright & Bonavida (31) who identified NK-resistant YAC variants that can bind NKCF and are susceptible to lysis by NKCF but are unable to stimulate production and/or release of NKCF. Of all the inhibitory agents tested, only phosphorylated mannose and antibody to granule cytolysin inhibited the binding of NKCF to target cells. As indicated in the section above, antibodies to LGL granule cytolysin presumably inhibit the activity of cytotoxic molecules after their release from the effector cells and prior to effective interactions with targets. The inhibition of binding of NKCF also by mannose-PO_4 suggests that a carbohydrate-dependent interaction is involved in the binding of NKCF to target cells. (See section on Binding of NKCF for more detailed discussion.)

The evidence for involvement of both LGL granule cytolysin and NKCF

Table 5 Effects on NKCF of agents that inhibit NK lysis[a]

Agent	Effect on			Reference
	Production and/or release	Binding	Lysis	
Sugar-PO4				
Mannose-PO4	—	↓	—	16, 72
Fructose-PO4	—	—	—	16, 72
Antigranule cytolysin	?	↓	—↑	16, 74
Protease inhibitors	—	—	—	16, 74, 75
Removal of Ca^{++}/Mg^{++}	↓	?	?	16, 75
Strontium chloride	↓	—	—	16
ATP or cAMP	↓	—	—	16
PGE_2	↓	—	—	16
Ammonium chloride	↓	—	—	16
Monensin	↓	—	↓	16

[a] The "—" indicates no change, ↑ indicates increase, and ↓ is decrease in NKCF production, binding, or lysis, ? indicates not possible to evaluate.

in NK activity raises the question of whether these molecules are related. The hypothesis of a direct relationship was supported by the ability of antibodies produced against purified cytoplasmic granules from LGL also to neutralize the activity of NKCF (74). However, from limited evidence, it is difficult to reconcile the major differences in their apparent molecular weights, their patterns of activity against various target cells, and in the kinetics of their lytic effects on target cells. Furthermore, it is possible that they are distinct molecules and that the apparent antigenic cross-reactivity is due to antibodies with various specificities in the polyclonal rabbit antisera to LGL granules. Alternatively, LGL granule cytolysin and NKCF may share portions of the same molecule. For example, upon release of LGL granule cytolysin into the culture medium, it may be degraded from the original 60-kd molecule into fragments of 20 and 40 kd, and such degradation may be accompanied by a marked decrease in the efficiency of its lytic activity. This hypothesis would not easily account for the NK-related target specificity of NKCF, in contrast to the much wider spectrum of activity of LGL granule cytolysin. However, it is possible that the portion of the cytolysin molecule that is responsible for the potent nonselective activity is lost or inactivated upon degradation to NKCF. To resolve this question, it will be very helpful to purify both proteins to homogeneity, to compare their amino acid sequences, and also to produce monoclonal antibodies against each.

Binding of Cytotoxic Molecules to Target Cells

BINDING OF GRANULE CYTOLYSIN As indicated above, LGL granule cytolysin releases the marker carboxy-fluorescein from liposomes in a rapid, calcium-dependent manner (76). Electron microscopic observations of negatively stained liposomes exposed to cytolysin and calcium showed cylindrical, pore-like structures inserted into the lipid bilayer. It was therefore proposed that after secretion from cytoplasmic granules, cytolysin inserts into a lipid bilayer where it forms pores. This mechanism would be similar to that proposed for the lytic activity of the terminal complement pathway (77; see also The Membrane Attack Complex of Complement by H. J. Müller-Eberhard, this volume), and to several bacterial toxins, including staphylococcal α-toxin (78) and streptolysin 0 (79).

To provide further insights into the nature of the interaction between cytolysin and lipid bilayers, researchers studied the effects of a number of different compounds on the lysis of target cells by LGL tumor granules (80). Table 6 is a summary of the data obtained in typical inhibition experiments with SRBC and YAC-1 targets. The lytic activity of granule cytolysin was inhibited by inorganic phosphate and by various monophosphoesters with I_{50} values in the range of 8–20 mM. Choline phosphate was exceptionally

potent, with an I_{50} of 1.4 mM. In contrast to the inhibition by phosphate esters, the parent compounds, such as neutral sugars, glycerol, and choline, showed no detectable inhibition at 50 mM. A lysolipid bearing a short aliphatic chain (L-α-lysocaproylphosphatidylcholine) and some detergents (CHAPS) were inhibitory with I_{50} values in the range of 1 mM. Lysolipids with longer aliphatic chains (L-α-lysopalmitoylphosphatidylcholine), phospholipids as liposomes, and related lipid compounds also displayed potent inhibition of the hemolytic activity of LGL granule cytolysin, with I_{50} values in the range of 0.2–30 μM. Soluble globular proteins inhibited LGL granule cytolysin hemolytic activity with I_{50} of 0.1–0.4 mg/ml. However, lipoproteins were 2–3 orders of magnitude more potent inhibitors, with I_{50} values less than 1 μg/ml. These results lend support to a model

Table 6 Inhibition of LGL granule cytolysin

Compounds tested	I_{50} (mM)[a]	
	SRBC	YAC-1
Phosphate compounds		
Inorganic phosphate	12	nd[b]
Organic phosphate[c]	0.4–8	2–7
Monophosphate esters		
Glycerophosphate	10	>36
Choline phosphate	1.4	nd
Sugar phosphate[d]	9–21	>50
Glycerol	>50	nd
Choline	>50	nd
Neutral sugars[e]	>50	>50
Lipids and lipid-like compounds		
L-α lysocaproyl phosphatidylcholine	0.8	nd
L-α lysopalmitoyl phosphatidylcholine	2×10^{-3}	$>9 \times 10^{-3}$
CHAPS detergent	0.7	nd
Phosphatidylcholine liposomes	0.2×10^{-3}	nd
Lipoprotein fraction of FCS	$<1 \times 10^{-3}$	1.5×10^{-3}
Globular proteins		
Bovine serum albumin (BSA)	0.4	2.7
Bovine gamma globulin (BGG)	0.2	2.5
Ovalbumin	0.1	>6

[a] Concentration of inhibition giving 50% reduction in granule cytolysin activity.
[b] nd, not done.
[c] ATP, GTP, RNA, and DNA.
[d] Glucose-1 and glucose-6-phosphate, fructose-1 and fructose-6-phosphate and mannose-6-phosphate.
[e] Glucose, fructose, mannose, galactose, N-acetylglucosamine, and N-acetylgalactosamine all give similar results.
(Reference 80.)

in which soluble cytolysin molecules insert into membrane lipid during the course of the lytic event.

BINDING OF NKCF Evidence for the presence of receptors for NKCF on the surface of NK-susceptible target cells was first obtained in experiments by Wright & Bonavida (68, 71), who demonstrated that the activity of NKCF preparations could be reduced or eliminated upon incubation with a variety of target cells. This loss of NKCF activity from the medium was most efficient when NK-susceptible target cells were used; however inhibitory effects were also seen with some NK-nonsusceptible targets. Because this type of experiment could not rule out the possibility of nonspecific binding to target cells or of inactivation of NKCF, e.g. by target cell–associated degradative enzymes, it was important to determine whether the target cells used to remove NKCF activity went on to lyse after removal of the original NKCF-containing medium. Such experiments showed that target cells incubated for 6 hr with NKCF and then washed were subsequently lysed with the same kinetics as when they were continuously exposed to NKCF (74, 81). The presence of mannose phosphate during the 6-hr incubation with NKCF blocked the subsequent lysis of the NK-susceptible target cells. After removal of the sugars by dialysis, the supernatants retained NKCF activity. These results support the hypothesis that the sugar molecules compete with carbohydrate determinants on NKCF for binding to receptors on the target cells. Alternatively, the inhibition by sugars could be explained by a lectin-like activity of NKCF, binding to phosphorylated sugars on the surface of target cells. In order to evaluate this latter possibility, we studied the effects of NKCF on target cells treated with tunicamycin, which blocks glycosylation of cell membranes by selectively inhibiting the addition of asparagine-linked sugars (74). Such treatment significantly decreased target cell susceptibility to lysis by NKCF and also decreased their ability to adsorb NKCF. In parallel, there was a significant decrease in the susceptibility of these tunicamycin-treated target cells to be lysed by purified LGL. To further test the hypothesis that NKCF might bind in a lectin-like fashion to mannosephosphate residues on the surface of target cells, the binding of NKCF preparations to mannose phosphate column was studied (16, 74). Inhibition by mannose saccharides of NKCF adsorption could be demonstrated, and the pattern of sugar inhibition seen was similar to that seen for inhibition of NK activity, i.e. mannose-6-phosphate inhibited activity, whereas nonphosphorylated mannose did not. Furthermore, tunicamycin-treated target cells demonstrated deficient NKCF binding and lysis.

Overall, the concept that NKCF binds to a glycoprotein or glycolipid receptor is consistent with the sugar inhibition data. Elucidation of the

chemical nature of the NKCF receptor must await binding studies performed with radiolabeled pure or recombinant NKCF. However, the events that occur after initial binding of NKCF still remain obscure.

Lysis of Target Cells

There is very little information on the mechanisms by which target cells are lysed by cytolytic molecules and, conversely, how they resist damage by NK cells. The hypothesis of the direct lytic effects of pore insertion in the target-cell membrane by LGL granule cytolysin remains to be experimentally supported, and virtually no information is available regarding the mechanism of lysis by NKCF. We focus here on the question of what factors seem to make target cells resistant to lysis.

There are several possible reasons: (*a*) target cells may not be recognized by NK cells; (*b*) they may not stimulate the release of cytotoxic factors from NK cells; and (*c*) they may not bind the factors that are released. Some recent data suggest that at stages following binding, some target cells are inherently resistant to lysis. The data come from two types of experiments: (*a*) cold-target inhibition of LGL granule cytolysin activity and (*b*) enzymatic treatment of target cells to modify resistance to lysis.

To investigate whether the ability to bind cytolysin correlated with susceptibility to lysis, a cold-target inhibition assay was developed to study quantitatively the competition of various target cells for LGL cytolysin (C. Reynolds, unpublished observations). As shown in Table 7, for the target cells tested, an inverse correlation was found between the amount of cytolysin required to give 50% lysis and the number of cells needed to cold-target-inhibit 10 units of LGL cytolysin. This relationship is especially obvious using SRBC, where only very small amounts of LGL cytolysin (1 unit) are necessary for 50% lysis, but large numbers of cells (1.7×10^8)

Table 7 Sensitivity to lysis and ability of various tumor cells to cold-target inhibit the cytotoxic effects of LGL granule cytolysin

Target cell	Units of cytolysin to give 50% lysis[a]	Number of target cells required to give 50% inhibition of LGL cytolysin
SRBC	1	1.7×10^8
RDM-4	51	1.4×10^4
YAC-1	159	1.5×10^4
EL-4	264	1.2×10^4
L5178Y	>1000	0.7×10^4
P815	>1000	0.6×10^4

[a] Various amounts of LGL granule cytolysin were added to 5×10^4 ^{51}Cr-labeled targets. One unit of LGL granule cytolysin is defined as the amount of material necessary to give 50% lysis of SRBC targets.
[b] Unlabeled target cells were added to 5×10^4 ^{51}Cr-labeled SRBC and 10 units of LGL granule cytolysin.

are needed to give 50% cold-target inhibition. The other target cells required substantially more LGL granule cytolysin to produce lysis, yet far fewer cells were necessary to give cold-target inhibition. These data indicate that those targets that are the best competitive inhibitors of LGL cytolysin are not the best targets. The most likely explanation for these observations is that the cytolysin-resistant targets are good cold-target inhibitors because they bind high amounts of the LGL cytolysin but are poor targets for lysis because of their ability to inactivate the cytolysin or to repair cytolysin-induced damage.

To investigate whether there is a cell-surface molecule involved in resistance to lysins, LGL cytolysin-sensitive and -resistant target cells were pretreated for 60 min with 1 mg/ml pronase and then tested for susceptibility to lysis by LGL cytolysin. As shown in Table 8, treatment of relatively sensitive target cells (SRBC, YAC-1) with pronase had no effect on the cytotoxic activity observed. In contrast, pretreatment of cytolysin-resistant target cells (P815, L5178Y) with pronase significantly increased their sensitivity to lysis. This change in susceptibility was not accompanied by a change in their capacity to bind cytolysin competitively (data not shown).

Similar experiments have been done with human NKCF and IFNα or IFNγ treatment of target cells (82). Treatment of K562 target cells with IFNγ markedly inhibited their lysis by NKCF and caused a 35–75% reduction in NK susceptibility, but did not significantly alter conjugate formation with LGL.

Taken together, such experiments suggest that some target cells may be inherently more resistant than other targets to the lytic effects of LGL granule cytolysin or NKCF. This resistance is independent of the competitive binding of cytolysin by these targets and appears to be at least partially due to surface molecule(s) which, when removed by pronase

Table 8 Effect of pronase treatment on target-cell susceptibility to lysis by LGL cytolysin

Amount of cytolysin (U) added	Pretreatment of target cells[a]							
	SRBC		YAC-1		P815		L5178Y	
	PBS	Pronase	PBS	Pronase	PBS	Pronase	PBS	Pronase
100	85[b]	81	41	43	11	57	11	24
25	87	84	27	27	5	28	7	19
6	82	83	23	18	3	15	4	12
1	70	72	17	12	1	4	NT	NT

[a] 1 mg/ml pronase for 60 min at 37°C.
[b] Percentage ^{51}Cr released from target cells upon incubation for 60 min at 37°C.

renders these cells more sensitive to lysis by LGL granule cytolysin. These data suggest that the surface characteristics of a target cell play an important role in ultimately determining its ability to be lysed by the cytotoxic molecules associated with NK cells.

RELATIONSHIP OF NKCF TO OTHER CYTOTOXIC OR CYTOSTATIC EFFECTORS

Recently, interest has centered on proteins isolated from activated leukocyte populations that have either cytolytic or cytostatic activity against tumor cells. For example, most interferon species inhibit the growth in vitro of a variety of tumor cell lines (83–86). In addition, a wide variety of other proteins derived from various activated effector cells have cytolytic or cytostatic activity (87, 88). Most of these factors have not been fully characterized or purified, so whether there are distinct protein mediators for the cytotoxic effects associated with each of the wide range of cytotoxic effector cells is uncertain. With the availability of an increasing number of genetically engineered homogeneous recombinant molecules with either cytostatic or cytolytic activity, and monoclonal and/or polyclonal antibodies against these defined proteins, it is now possible to analyze the role of these factors in the various forms of cell-mediated cytotoxicity. Our studies have particularly focused on the possible relationship of such defined cytotoxic molecules to NKCF and/or granule cytolysin and on their possible involvement in the cytolytic and/or cytostatic activity of NK cells or other natural effector cells. Particular emphasis has been placed on the possible relationship of lymphotoxin (LT) or tumor necrosis factor (TNF) to NKCF.

Lymphotoxin was initially derived from cell supernatants of antigen- or mitogen-stimulated lymphocytes and has been reported to have tumoricidal or cytostatic activity in situ against a wide range of tumor cells (87). It has also recently been reported to interfere with the malignant transformation of cells (89, 90). LT appears to be produced mainly by stimulated T cells or B cells (91, 92) and has also been found to be constitutively produced by some human B lymphoblastoid cell lines (92). TNF has also been shown to have cytostatic or cytolytic activity against a variety of transformed cell lines in vitro, whereas most normal target cells have been resistant to its effects (93–95). In addition, TNF has an impressive ability to produce extensive hemorrhagic necrosis of several mouse tumor cell lines in vivo, particularly after intralesional injections (93, 94, 96). In contrast to LT, TNF appears to be produced mainly by macrophages or macrophage cell lines (94, 95). The cloning of the genes for LT and TNF in *Escherichia coli* has demonstrated about 30% amino acid homology between the two molecules (97).

Lymphotoxin and TNF are active against a much narrower range of target cells then those that are NK-sensitive, with L929 perhaps the most susceptible target. The majority of NK-susceptible human and mouse targets are not susceptible to growth inhibition or cytolysis by either of these recombinant molecules (J. Ortaldo, R. B. Herberman, unpublished observations). In addition, the cells producing these factors and the stimuli needed to induce them vary considerably. TNF and LT are not produced by NK cells and are mainly produced by monocyte or B lineage cells. Antigen and lectins are the usual stimuli for release of TNF and LT (87, 91, 94), whereas NK targets are the best stimuli for release of NKCF (16, 68, 71).

The mechanism by which LT and TNF affect target cells is also distinct from NKCF or LGL granule cytolysin. For example, whereas mannose-PO_4 inhibits lysis of targets by NKCF (16, 74), it has no effect on the activity of either LT or TNF (J. Ortaldo, R. B. Herberman, unpublished observations). In addition, whereas tunicamycin pretreatment of target cells inhibits binding to NKCF and subsequent lysis by this factor, such treatment enhances the susceptibility to lysis by TNF (J. Ortaldo, R. B. Herberman, unpublished observations).

To examine the relationship of the factors more directly and to determine their role in NK cell–mediated cytotoxicity, studies have been performed with antibodies to recombinant human LT and TNF (98). A summary of these results is present in Table 9. The antibodies to LT or TNF strongly inhibited the cytotoxicity by the homologous protein against L929 cells but had no detectable effects on the cytotoxicity induced by NKCF or on NK or ADCC activities. For comparison, the effects of antibodies to granule cytolysin were examined in the same experiments. The anticytolysin, as expected, inhibited the cytotoxicity by NKCF and also inhibited NK and ADCC activities, but had no effects on the activity of either LT or TNF. These results support the conclusions that NKCF and LGL granule cytolysin are distinct from LT or TNF and that their activity does not depend on the presence of either LT or TNF in the assay. Further, ADCC or NK activity against the prototype target cells appears not to be mediated by, or dependent on, either LT or TNF.

It is possible that NKCF is not one factor, but a functional activity that is the sum of many factors acting synergistically. However, NKCF activity could not be mimicked by various combinations of LT, TNF, and α or γ interferon. Therefore, it seems very unlikely that LT, TNF, or interferon, or a combination of these molecules are involved in the cytotoxic activity mediated by NKCF.

Coincubation of human lymphocytes with NK-sensitive target cells causes release of a cytostatic factor into the culture medium (98). Since this procedure was initially described for the production of NKCF, it was

Table 9 Effects of antibodies to LT, TNF, or granule cytolysin on cytolysis by effector cells or by cytotoxic molecules

Components of interaction	Effector/target combinations and their effects					
Effectors	PBL or LGL	PBL or LGL ab-target	NKCF	Granules	LT	TNF
Targets	K562	(ADCC)	K562	YAC-1	L929	L929
Antibody added to assay						
Rabbit antirat LGL granule cytolysin	↓[a]	↓	↓	↓	–	–
Monoclonal antirecombinant human LT	–	–	–	–	↓	–
Monoclonal or rabbit antirecombinant human LT	–	–	–	–	–	↓

[a] ↓, significant inhibition of cytolysis; –, no significant effect; ↑, significant augmentation of cytolysis; ?, not done or not interpretable.

important to evaluate the role of NKCF in this growth inhibition. It also was of interest to determine the possible relationship of this cytostatic factor to leukoregulin, a molecule with cytostatic activity reportedly induced from human PBL by incubation with lectins (99). As demonstrated earlier, NKCF has a somewhat limited target specificity and lyses only NK-sensitive target cells. In contrast, leukoregulin and the cytostatic factor induced by target cells were highly cytostatic for a number of cells like the mouse lymphoma, MBL2, which has little sensitivity to lysis by human NK cells. The effects of mannose-6-phosphate and antibodies against rat LGL granule cytolysin were also tested. These reagents, as discussed above strongly inhibited NKCF but had no effect on the ability of either leukoregulin or target cell–induced cytostatic factor to mediate growth inhibitory effects on a variety of targets (data not shown).

The fact that antibodies to recombinant lymphotoxin and TNF failed to inhibit cytostasis by leukoregulin or target cell–induced cytostatic factor suggests that LT or TNF are not involved in the growth-inhibitory activity against K562. In addition, the growth of K562 was not affected by high concentrations of lymphotoxin or TNF. The failure of antibodies to LT or TNF to inhibit the antiproliferative effects of leukoregulin or tumor cell-induced cytostatic factor also argues against a possible synergy between low levels of LT and TNF and the other cytostatic factors.

Lymphotoxin has been reported to have a molecular weight of 18 kd (96), and TNF has an apparent M_r of 17 kd (95, 97). Although the exact molecular weights of NKCF, leukoregulin, or tumor-induced cytostatic factor have not been determined, preliminary evidence indicates that NKCF has an M_r of 18 kd, and both leukoregulin and tumor-induced cytostatic factor have M_r of 20 kd (99, 100; T. Sayers, J. Ortaldo, R. B Herberman, unpublished observations).

From all evidence, it appears that the tumor cell–induced cytostatic factor and leukoregulin represent the same factor, which is distinct from NKCF, LT, or TNF. This factor can be induced from PBL by either NK-sensitive target cells or lectins, but further studies are needed to determine whether it is produced by NK cells and/or by other effector cells.

ROLE OF TNF IN CYTOTOXICITY BY NATURAL CYTOTOXIC (NC) CELLS

In studies on the possible involvement of TNF or LT in cytotoxicity by NK cells, it was of considerable interest to examine the possible involvement of these factors in cytolysis by NC cells. NC cells have been detected in mice as natural effector cells which differ from NK cells in regard to target selectivity, cell-surface characteristics, organ and strain distribution, and

susceptibility to regulatory factors (101). Whereas the prototypic target cell for detection of mouse NK activity is the YAC-1 lymphoma cell line, WEHI-164 cells are the main targets used to measure NC activity selectively (101, 102). Elimination by antibody plus complement of cells bearing any of the markers that have been associated with mouse NK activity has little effect on NC activity (101–103). Whereas NK activity reaches close to maximal levels within 4 hr, NC activity usually requires incubation periods of 18 hr or longer (101–103). While NK activity is augmented by pretreatment of the effector cells with interferon or interleukin 2, NC activity is enhanced primarily by interleukin 3 (104). Also, while NK cells have been closely associated with LGL, it has been difficult to associate NC activity directly with any specific cell type, suggesting that it is not a cell-mediated event. This possibility is also suggested by observations that NC-susceptible targets, such as WEHI-164, are often lysed at very low ratios of effector to target cells, frequently below 1 : 1. We therefore considered the possibility that this form of effector activity was mediated by release of a soluble factor, and we examined the possible involvement of TNF and LT (105).

TNF was of particular interest as a possible mediator of NC activity since the prototype target cell for NC activity, WEHI-164, is highly susceptible to lysis by recombinant mouse, as well as by human, TNF. Table 10 summarizes the effects of antibodies against mouse TNF on mouse NC and NK activity as well as on the recombinant protein (105). Both antibodies completely neutralized the cytotoxic effects of recombinant mouse TNF on the WEHI-164 target cells and completely inhibited the NC activity of normal spleen cells against WEHI-164, but had no detectable effect on NK

Table 10 Cytotoxicity of TNF against WEHI-164 cells and effect of anti-TNF on NC activity of normal mouse spleen cells[a]

Factor of effector cells	Antibody	Lytic activity versus	
		WEHI-164	YAC-1
Mouse rTNF[b]	None	+	−
Mouse rTNF	Antimouse rTNF	−	−
Mouse rTNF	Antimouse TNF	−	−
Spleen cells (C57BL/6)	None	+	+
Spleen cells (C57BL/6)	Antimouse rTNF	−	+
Spleen cells (C57BL/6)	Antimouse TNF	−	+
Spleen cells (C57BL/6)	Antihuman rLT	+	+

[a] Summary of data from (105).
[b] rTNF, recombinant TNF.

activity against YAC-1 target cells. Thus, in contrast to NK activity, mouse NC activity depends on the release of TNF during the cytotoxic assay. Whether the release of this cytotoxic molecule is spontaneous or is triggered by the interaction of the effector cells with the NC-susceptible target cells is unclear. The association of TNF release with NC activity should also provide a new basis to characterize the NC cell. In view of the previous association of TNF production with macrophages (94, 95), these results suggest that the NC cell may be within the myelomonocytic lineage.

SUMMARY AND COMPARISON WITH MECHANISMS OF CYTOTOXICITY BY T CELLS AND MACROPHAGES

The initial phase in the interaction of NK cells with NK-susceptible target cells is the recognition of target structures by receptors on the surface of NK cells. Little is known about the nature of this receptor, but it seems to be distinct from the T cell receptor. The target structures recognized by NK cells are also poorly understood but again are different from those recognized by the T cell–receptor complex. A fundamental distinction between recognition by NK cells and T cells is the apparent lack of involvement of recognition of the major histocompatibility complex by the natural effector cells. Indeed, some of the most sensitive targets for NK activity are devoid of antigens of the major histocompatibility complex.

The phases subsequent to recognition and conjugate formation and leading to target lysis appear very similar, if not identical, for NK cells and T cells. Agents that interfere with postbinding steps of NK activity also inhibit T cell–mediated cytotoxicity, and substantial evidence has been gathered for the involvement of a granule cytolysin in T cell–mediated lysis (106). The characteristics of T-cell granule cytolysin are very similar to those described for LGL granule cytolysin, and these proteins may be closely analogous, if not entirely identical.

About the mechanism of cytotoxicity by macrophages, considerably less information is available. However, interaction between cytotoxic macrophages and tumor target cells appears to be similar to that used by NK cells or CTL (107). Activated macrophages bind to susceptible targets by receptors able to discriminate accurately between tumor cells and normal cells. However, there is virtually no information about these receptors or about the target structure they recognize on tumor cells. Several different mechanisms for the lytic process have been explored. The possible role of reactive oxygen species in the mechanism of cytotoxicity by macrophages has been carefully examined, since activated macrophages can undergo a vigorous oxidative burst and this mechanism appears to contribute to

antibody-dependent macrophage-mediated cytotoxicity of erythrocytes (108). Involvement of reactive oxygen species has also been suggested for NK activity (109), but several recent studies have indicated that purified LGL cannot undergo an oxidative burst (e.g. 110). Rather, Greenberg et al (111) have found that NK cells can be stimulated by NK-susceptible targets to release a factor that stimulates macrophages to undergo an oxidative burst.

As with NK cells and CTL, there is substantial evidence for an involvement of proteases in the killing of tumor cells by macrophages (107). In fact, it has been suggested that macrophages release a protease that is selectively cytolytic for tumor cells (107). However, this enzyme has not yet been purified, and it remains unclear whether it directly causes lysis or is involved in a cascade of events leading to the generation of cytolytic molecules.

In summary, there are many details of the mechanisms responsible for cell-mediated cytotoxicity that remain to be defined. However, the discovery of granule cytolysins and the cloning of soluble cytolytic molecules such as TNF and LT have allowed rapid progress in the understanding of the potential effector molecules. It may be anticipated that the characterization of NKCF and leukoregulin and the determination of the nature of the structures involved in recognition will provide the next major advances in our understanding of the mechanism of cytotoxicity by NK cells.

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CONTENTS

CANCER IMMUNOTHERAPY USING INTERLEUKIN-2 AND INTERLEUKIN-2-ACTIVATED LYMPHOCYTES[1]

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INTRODUCTION

The development of immunologic approaches to the treatment of cancer has progressed through major changes in the past five years. In the 1970s considerable enthusiasm existed for immunotherapeutic approaches to cancer based on attempts to provide nonspecific stimulation of the host immune system in the hope that this general increase in immune reactivity would lead to an increased reactivity to putative tumor antigens on growing cancers. This approach was based on minimal experimental data from animal tumor models. A large number of clinical trials in humans utilized attempts to stimulate the immune system nonspecifically. Using substances such as Bacillus Calmette-Guerin (BCG), methenol-extracted residue (MER), Corynebacterium parvum, levamisole, and other immune stimulators, researchers treated patients with extensive tumor, as well as with minimal tumor burdens. This experience was almost universally unsuccessful and, both in animal tumor model systems and in the human, has largely been abandoned (1).

An alternate approach to cancer immunotherapy can be categorized as "passive" or "adoptive" immunotherapy, in which the tumor-bearing host receives the systemic transfer of immunologic reagents such as antibodies or reactive cells already possessing antitumor reactivity (2–5). Because the

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cellular effector arm of the immune system is primarily responsible for the rejection of both allografted and tumor tissue, increasing emphasis has been placed on this cellular transfer approach in recent years. A variety of animal models have shown that the transfer to the tumor-bearing host of relatively large numbers of sensitized lymphoid cells with a high degree of specific antitumor reactivity can mediate the rejection of established localized and metastatic cancer (2–10).

The major impediment to the development of a successful adoptive immunotherapy for the treatment of established cancer in humans has been the inability to generate lymphoid cells with antitumor reactivity. The description of interleukin-2 (IL-2) in 1976 (11), however, opened several new possibilities for this approach. The advent of IL-2 made it possible to expand T-lymphoid cells in culture, and the possibility existed that even a small number of lymphoid cells with antitumor reactivity could be expanded into numbers sufficient to mediate antitumor effects. In 1980, a rather unexpected finding opened yet further possibilities for the generation of lymphoid cells with antitumor reactivity. In both the mouse and the human, the incubation of normal lymphocytes in IL-2 was found to result in the generation of cells capable of killing fresh tumor target cells, but not normal cells, in short-term chromium release assays (12, 13). These lymphokine-activated killer (LAK) cells have been extensively characterized and appear able to mediate antitumor effects in vivo (14–18). Administration in vivo of IL-2 could lead to the in vivo expansion of cells with antitumor reactivity (19, 20). Further, the administration of sufficient quantities of IL-2 resulted in the generation of LAK cells in vivo that probably mediate antitumor reactivity directly (21, 22). Studies in this area have received great impetus from the cloning of the gene for IL-2 (23) and its successful insertion and expression in *Escherichia coli*. Thus, large amounts of highly purified IL-2 with full biologic activity are now available for use in animal and human studies (24).

The use of IL-2 and IL-2-activated lymphoid cells (LAK cells) for the immunotherapy of animal and human tumors has progressed significantly in recent years. We review results with the use of these approaches in the immunotherapy of a variety of established tumors in mice and in humans.

INTERLEUKIN-2 AS A PHARMACOLOGIC REAGENT

In Vitro Evidence for a Pharmacologic Role of IL-2

Numerous studies in rodents and humans demonstrated that decreased immune responsiveness in vitro can be reversed by supernatants containing IL-2 (summarized in Table 1). Following incubation in IL-2, murine

thymocytes produce a cytolytic response almost equal to that of peripheral T cells. In addition, peanut agglutinin positive thymocytes, thought to represent immature cortical cells, generate cytolytic activity in the presence of IL-2. Further extension of these experiments in vitro and in vivo demonstrated that a number of functions normally poorly expressed, if at all, in nude mice developed with the administration of IL-2 in vivo, including the cytolytic response to alloantigens (25), a helper-T-cell response against heterologous erythrocytes (26), and the generation of autoantibodies (27).

Aged animals exhibit diminished IL-2 production as well as decreased allogeneic mixed lymphocyte responses, cell-mediated lympholysis, and antigen-induced T-cell proliferation. As measured in these immune assays T-cell function was markedly enhanced by supplementing cultures with supernatants containing IL-2 (28).

Immunosuppressive treatment with agents such as cyclosporin A or cyclophosphamide leads in animals to a marked diminution or failure

Table 1 In vitro evidence for a possible pharmacologic role of IL-2

Murine and rat studies
Decreased immune responsiveness is reversed by IL-2 containing supernatants
Nude mice (25–27)
Aged animals (28)
Cyclosporine-treated animals (29)
Cyclophosphamide-treated animals (30)
IL-2 containing supernatants and purified IL-2 allows generation of lymphokine-activated killer cells
Murine IL-2 from Con A–induced spleen cells or EL-4 tumor (12, 16)
Recombinant human IL-2 (24)
IL-2 allows the differentiation of cytotoxic T cells (31)
Metabolically inactivated cells (32)
Reverses inhibitory effect of antibody to IL-2 (33)
Human studies
Immune deficiencies and immune responsiveness can be partially restored by IL-2
Acquired immunodeficiency syndrome (34)
Other immunodeficiencies including Nezeloff's syndrome (35)
Leprosy (36)
Cancer (37)
Rheumatoid arthritis (38)
IL-2 allows generation of lymphokine activated killer cells
PHA-activated lymphocyte supernatants (13)
Human tumor–derived (Jurkat) IL-2 (54)
Recombinant IL-2 (22)
IL-2 stimulates the migration of activated T cells (39)
IL-2 allows the differentiation of metabolically inactivated cells into cytotoxic T cells (40)

to respond significantly to alloantigen (29, 30). Addition of exogenous sources of IL-2 was found to result in a marked increase in reactivity. Cyclophosphamide-treated mice have adequate CTL precursors to mount an allogeneic cytotoxic-T-cell response but lack sufficient Lyt^{1+2-} cells. The supernatants of mixed lymphocyte cultures, containing IL-2 as well as other lymphokines, were capable of reversing this defect in cyclophosphamide-treated mice. Thus, in a number of situations associated with decreased immune responsiveness, supernatants containing IL-2 used in vitro were capable of reversing this immune suppression.

In addition to the ability of IL-2 to mediate the generation of LAK cells in vitro, IL-2 also appears important in the differentiation of cytotoxic T cells. The stimulating role of metabolically inactivated (heat-inactivated or UV-irradiated) cells can be reconstituted by the addition of supernatants containing IL-2 (31, 32). In addition, when antibodies to IL-2 are added to mixed-leukocyte-reaction cultures, an effective decrement in T-cell division occurs, as assessed by tritiated thymidine incorporation and by short-term cytoxicity assays. These effects could be reversed by adding IL-2 to the culture (33).

A variety of human immune deficiencies, demonstratable by in vitro assays, can also be partially restored by the addition of supernatants containing IL-2. When taken from patients with the acquired immunodeficiency syndrome (34), lymphocytes incubated with IL-2 demonstrated an increased ability to lyse both virally infected target cells and the cultured tumor cell line K562. The reconstitution of in vitro reactivity of cells from patients with immune defects such as Nezoloff's syndrome (35), patients with leprosy (36), cancer (37), and rheumatoid arthritis (38), has been demonstrated in all cases by adding IL-2. In vitro mixed lymphocyte culture of cells from a child with Nezoloff's T-cell deficiency showed marked proliferative responses to T-cell mitogens and alloantigens and the generation of cytotoxic cells only when exogenous IL-2 was added (35).

Patients with leprosy have deficient T-cell responses to *M. leprae* but not to other antigens (36). This selective antigen defect appears to occur at the T-helper-cell level since cells are capable of proliferating to this antigen when exogenous IL-2 is added to cultures. The ability of patients with rheumatoid arthritis to respond in vitro with a proliferative response to PPD was significantly depressed compared to other individuals (38). This could not be reversed by prostaglandin inhibitors such as indomethicin but was overcome by addition of crude supernatants from a mixed lymphocyte reaction as well as by purified IL-2, but not by IL-1.

Recent experiments have indicated that IL-2 is a potent inducer of human-T-lymphocyte motility (39). Unstimulated T cells do not respond to IL-2 alone, but T cells exposed to mitogens respond with significantly

increased migration. This activity appeared to be primarily limited to the OKT4+ subset and thus suggested that IL-2 may serve as a specific regulatory molecule causing the proliferation *and* specific migration of class-II-reactive T cells.

Finally, in observations similar to those made in murine studies, it was discovered that IL-2 restores the generation of target-specific CTL by providing a proliferative stimulus when allogeneic stimulator cells had been rendered nonimmunogenic by UV irradiation or by heat treatment (40). Thus, IL-2 administration could potentially be useful in vivo for increasing the immunogenic response to weak antigens.

In Vivo Administration of IL-2 in Rodents (Table 2)

The earliest studies demonstrating an in vivo effect of IL-2 were conducted in nude mice. In studies reported by Wagner and colleagues, Con-A-stimulated spleen cell supernatants were injected into nude mice along with allogeneic stimulator cells (25). This treatment allowed the in vivo differentiation of alloreactive cytotoxic T lymphocytes. In addition, rat erythrocytes injected along with IL-2-containing supernatants, into nude mice resulted in the development of autoantibodies to murine erythrocytes (27). These studies were the first clear indication that IL-2 might have an in vivo pharmacologic role and in fact partially defined the immune defect present within nude mice.

In specially prepared B rats, treated with 750 rads of irradiation following adult thymectomy and reconstituted with syngeneic bone marrow cells, cardiac allografts survived indefinitely (41). Transferred alloimmune syngeneic spleen cells alone were incapable of causing graft rejection. These animals developed acute graft rejection when IL-2-enriched, conditioned supernatant was injected along with transferred cells. In this early study, little information was provided regarding the precise dose of IL-2 required to demonstrate this effect. Conditioned media supernatants were given in a volume that was 20% of the circulating blood volume. These supernatants were given intravenously for 5 consecutive days, and when given alone, did not cause the rats to develop graft rejection. Similarly, an antibody to the IL-2 receptor was noted to prolong cardiac allograft survival in mice indicating that IL-2 itself is important in the rejection process (42).

IL-2 administration can stimulate an increase in the in vivo cellular cytotoxic response to alloantigens (43, 44) when IL-2 is given 1 to 2 days following allostimulation. Similarly in immunosuppressed animals treated with cyclophosphamide, an allocytotoxic response could be reconstituted by the in vivo injection of MLC supernatants containing IL-2 (45) at the site of a draining inguinal node adjacent to the alloimmunization site.

Table 2 Effects of the in vivo administration of IL-2 to rodents

Effect	Reference	Source and dose of IL-2	Strain	$T_{1/2}$ IL-2	Toxocity	Comment
Restore immunity in nude mice	25	Con A–stimulated spleen cells, iv, units NS[a]	B6 nu/nu	NS[a]	NS	Increased alloresponse (cytotoxicity)
	26	Same	B6 nu/nu	NS	NS	Induction of T helper cells for RBC
	27	Same	C3H & BALB/c nu/nu	NS	Hemolysis	Development of auto-antibodies to erythro-cytes
Causes rejection of stable cardiac allografts with transferred cells	41	Con A–stimulated rat spleen cell supernatants, iv, units NS[a]	Lewis rat	NS	NS	IL-2 alone did not cause rejection of stable cardiac grafts
Increase alloimmune response	43	PMA-stimulated EL-4 or Con A–stimulated splenocytes ip tid 1400 units × 3 days	B6 & DBA/2	3–5″	Debility	Mice injected with IL-2 one day after immuniza-tion
	44	PHA-stimulated LBRM lymphoma; 10–100 U ip	BALB/c	NS	NS	IL-2 given 2 days after ip immunization worked better than concurrent administration
Increase natural killer activity	44	PHA stimulated LBRM lymphoma; 50–150 U ip	CBA/J	NS	NS	Tested in spleen cells 48 hr after ip treat-ment
	21	Human recombinant IL-2 (HRIL-2) 10^3–10^4 units ip, tid	B6	1.6″	NS	Tested in spleen cells after 3 days
In vivo generation of LAK	21	Human recombinant IL-2 (HRIL-2) 10^3–10^4 U iv, ip, or subcutaneous	B6	1.6″	NS	Continuous administration over 4 days caused LAK activity in spleen
	20	HRIL-2, 1–2 × 10^5 U ip tid × 5 days	B6	NS	NS	Lungs, liver, and mesenteric LN all with LAK activity

Increase growth and function of cultured transferred T cells	19	Purified PHA, PMA stimulated LBRM-33, 320 U ip daily × 5 days	B6	NS	NS	Long-term cultured T cells distinguished from host using congenic mouse cells
Increase proliferation of endogenous lymphocytes	20	HRIL-2, 1–2 × 10^5 U ip tid × 5 days	B6	NS	NS	Increased proliferation in lung, liver, spleen, kidney, mesenteric lymph nodes
	46	HRIL-2, 2 × 10^5 U/kg/hr iv by continuous infusion	Fisher rat	2.9″	Hepatotoxicity	Increased proliferation of lymphocytes in lung, liver, spleen; decreased in thymus
Increase immunity and clearance of viruses by transferred cells	47	Purified EL-4 supernatants iv or ip 100–200 U/day	C3H/HEJ mice	NS	NS	Antiviral activity of transferred anti-herpes specific T cells increased
Causes regression of pulmonary metastases and subcutaneous tumor	22	Purified HRIL-2, 5 × 10^3–10^5 U ip × 5 days	B6	NS	NS	Marked dose dependence requiring $>10^6$ U/kg in sarcomas and melanomas; effect radiation sensitive
Causes regression of liver metastases	48	HRIL-2, 1–25 × 10^3 U ip tid × 5 days	B6	NS	Ascites	*Hepatic sarcoma mets* reduced
Augments antitumor effect of long-term cultured T cells	49	Purified LBRM supernatants; 80 U ip daily × 5 days	B6	NS	None	Adoptive therapy of FBL-3 lymphoma
	50, 51	Purified human Jurkat derived EL-4 supernatants, 500 U ip in gelatin daily for 3 days	B6	1.6–2.6″	None	Adoptive therapy of FBL-3 lymphoma

[a] NS, not stated.

Mice treated with supernatants from the PHA-stimulated LBRM lymphoma or with human recombinant IL-2 (21, 44) display an increased ability to lyse NK-sensitive target cells. In both studies, increased natural killer cell activity was found in the spleen within 2 to 3 days following daily administration of IL-2.

IL-2 administered intraperitoneally can lead to the generation of LAK cells in vivo (21). In mice receiving 150,000–200,000 units of IL-2 three times a day, fresh lymphocytes obtained from lung, liver, spleen, and mesenteric lymph nodes exhibited significant cytotoxicity for fresh tumor target cells in short-term chromium-release assays (20).

When IL-2 derived from the LBRM tumor line was injected intraperitoneally at low doses (320 units/day) for 5 days in mice congenic at the Thy-1 allele, the experiment demonstrated that donor cells introduced into the peritoneal cavity expanded significantly (19). Donor cells in the ascites of treated mice increased by as much as 10-fold with IL-2 administration and as much as 20-fold in the spleen of irradiated or cyclophosphamide-treated mice. Increase in T-cell growth, augmented by IL-2, occurred only with T cells that had been precultured in IL-2.

In addition to the proliferation of transferred T cells under the influence of administered IL-2, the proliferation of endogenous lymphocytes has been demonstrated using an assay based on the incorporation of ^{125}labeled IUdR into whole mice (20). The increase in endogenous lymphocytes occurred in the lung, liver, spleen, kidney, and mesenteric lymph nodes of animals treated with 1–2 $\times$ 10^5 units of IL-2 intraperitoneally 3 times a day for 5 days, the proliferation was inhibited by irradiating the animals.

Using a continuous infusion of human recombinant IL-2 in rats, researchers studied the toxicity of IL-2 administration (46). Rats showed a half-life of HRIL-2 of approximately 2.9 min; this was similar to the half-life found in mice. Increased proliferation of lymphocytes within the lung, liver, and spleen of rats receiving IL-2 by this route was noted histologically. A decreased number of lymphocytes appeared in the thymus. Very high doses of IL-2 had significant toxicity that could lead to the death of affected animals. A dose of 140,000 U/kg/hr was uniformly lethal, while no deaths were noted in rats receiving 3,000 U/kg/hr.

Studies evaluated the role of IL-2 in augmenting immunity to herpes simplex virus in C3H/HEJ mice. These demonstrated that purified IL-2 from EL-4-stimulated tumor cells administered in conjunction with transferred antiherpes-specific T cells increased cell-mediated antiviral activity (47). Relatively low quantities of IL-2 administered intraperitoneally in gelatin decreased the mean viral titers 20–100 fold.

The systemic administration of high doses of recombinant IL-2 alone to tumor-bearing mice can lead to the generation of LAK cells in vivo and can

mediate the regression of established pulmonary hepatic and subcutaneous metastases from a variety of tumors (22). A mean reduction of pulmonary metastases of 80% was seen in 10 experiments involving mice treated with 100,000–170,000 units of IL-2, starting 3 days after tumor injection. The effectiveness of IL-2 was even greater in animals whose treatment was begun 10 days following establishment of pulmonary metastases. In these animals, doses of 20,000–50,000 units of IL-2 administered 3 times a day mediated tumor regression. These treatments appear to require the in vivo proliferation of lymphoid cells since irradiated mice received no benefit from IL-2 therapy. Similar experiments, using the MCA-105 sarcoma metastatic to the liver (48), demonstrated that IL-2 injected intraperitoneally 3 times a day for 7 days was effective in markedly reducing the number of liver metastases. In 20 consecutive experiments, various doses of IL-2 resulted in a consistent reduction in hepatic metastases at all doses over 15,000 units administered intraperitoneally 3 times a day.

Studies conducted by Cheever et al (49) and by Donohue et al (50, 51) have demonstrated that IL-2 can enhance the therapeutic effectiveness of the adoptive transfer of specifically immune, cultured T cells in mice bearing the FBL-3 lymphoma. Shu & Rosenberg have reported similar findings in mice bearing MCA-induced sarcomas (10).

In summary, in a number of different murine models, IL-2 significantly increased immune reactivity and the growth and function of both endogenous and transferred T cells, as well as of lymphokine-activated killer cells. In addition, at very high doses, IL-2 alone appeared to have significant antitumor effects. The mechanism by which IL-2 mediated these effects is thought to be primarily the ability of this agent to increase the proliferation of lymphoid cells as well as the generation, in vivo, of lymphokine-activated killer cells.

Studies of IL-2 Administered to Patients With Cancer (Table 3)

The first reported studies of the administration of IL-2 in humans used relatively low doses of IL-2 obtained from the supernatants of mitogen-stimulated normal human lymphocytes (52). Doses of 2–8.4 $\times$ 10^4 units of this material were administered to patients with malignancy. Increased spontaneous lymphocyte proliferation as well as the development of atypical lymphocytes in the circulation was reported.

We began our studies with IL-2 derived from the Jurkat cell line in 1983. A total of approximately 30 mg of IL-2 was purified from thousands of liters of supernatant from stimulated Jurkat tumor cells. The serum half-life, toxicity, and in vivo immunologic effects of this IL-2 preparation were studied in patients with AIDS, as well as in patients with cancer un-

Table 3 Pharmacokinetics, immunologic findings, and toxicity of IL-2 in patients with cancer

Source and dose of purified IL-2	Reference	$T_{1/2}$	Number of patients	Immunologic findings	Toxicity
PHA-stimulated human PBL (2–8.4 × 10^4 U)	52	22.5″	2	Increased spontaneous lymphocyte proliferation	Fever
				Occasional atypical lymphocytes and lymphocytopenia	Increased cortisol levels
					Mild DIC
Jurkat tumor derived IL-2 (Total dose of 5.6 × 10^4 U − 8 × 10^6 U)	53, 54	5–7″ (α) 30–120 (β)	16	Acute decrease in total PBMC after bolus administration	Fever, chills
				Decreased IL-2 responsiveness and ability to generate LAK from PBMC after approximately 15 min with recovery by 24–48 hr	Headache Malaise Transient hyperbilirubinemia
				Increase in serum ACTH and cortisol	
Recombinant IL-2 (Total dose of 0.4–2.5 × 10^6 U/kg)	56, 57	6.9″ (α) 70″ (β)	25	Early depletion of all lymphoid cells followed by expansion with continuous administration of IL-2	Fever, chills Headache Malaise Nausea and vomiting
				2–16-fold increase in total lymphoid cells with continuous IL-2 administration	Weight gain and fluid retention
				Increase in serum gamma interferon levels	Diarrhea Rash and pruritis
				Early depletion of LAK precursors	Eosinophilia
				Increase in Tac (+) cells in the periphery with continuous IL-2 administration	

responsive to standard therapy (53, 54). Patients received 0.25, 2.5, or 25 μg/kg of IL-2 by bolus or by 24-hour continuous infusion on a weekly basis for 4 weeks. The serum half-life of IL-2 was short, with an alpha distribution phase of 5–7 min and a beta clearance phase of 30–120 min. A total of 16 patients were studied, 4 of whom received the direct intralesional injection of IL-2. Immunologic findings associated with the use of Jurkat IL-2 included an acute decrease in the total numbers of peripheral-blood mononuclear cells obtainable after bolus administration, decreased IL-2 mitogenic responsiveness, and decreased ability to generate LAK cells from PBMC within 15 min following IL-2 administration, an ability recovered by 24–48 hr. An increase in serum ACTH and cortisol was noted. No patients demonstrated beneficial therapeutic effects, and toxocity was minimal including: headache (6/16), nausea (4/16), malaise (6/16), and fever and chills (8/16). No pulmonary, hematologic, or renal toxicity was noted. Our ability to conduct further studies with the use of this IL-2 preparation was limited by its availability. Thus, future studies were conducted with recombinant IL-2 (kindly supplied by the Cetus Corporation, Emeryville, Calif.).

The description of the cloning of the gene for IL-2 by Taniguchi et al in 1983 and its subsequent stable expression in *E. coli* allowed production of large quantities of IL-2 (23, 24). Site-specific mutagenesis of the human IL-2 gene, with a serine-for-cysteine substitution at amino acid residue 125 of the mature protein, allowed the production of a stable molecule containing the full biologic activity of native IL-2 (55). We have currently treated over 25 patients with recombinant IL-2 alone and have determined that its serum alpha distribution phase is 6.9 min, with a beta clearance phase of approximately 70 min (56). One of the difficulties associated with conducting serial immunologic assays in patients receiving IL-2 is that all lymphoid cells decrease in the peripheral blood following IL-2 administration. With continuous administration of IL-2, recovery and actual expansion of lymphoid cells in the periphery occurs, with the total numbers of lymphocytes sometimes increasing as much as 16-fold.

Measurement of IL-2 in the serum (Figure 1) is readily accomplished using the in vitro biologic assay initially described by Gillis and by Smith (54). An increase in gamma interferon levels, with up to 26 units of gamma interferon detectable in the serum at 2 hr, followed bolus infusion of 10^6 units/kg of IL-2. Interferon levels decreased to normal within 48 hr following treatment. Smaller increases of interferon occurred with continuous infusion of IL-2. In the use of both the Jurkat and recombinant IL-2, one of the most consistent findings is a marked early decrease in LAK precursors within the peripheral blood following bolus IL-2 administration. As shown in Table 4, patients receiving a variety of doses of IL-2

exhibited the early depletion of cells capable of generating LAK activity following IL-2 incubation. LAK precursor cells were rapidly cleared with decreases to less than 1–5% of the starting number of lytic cells within minutes following the administration of IL-2. Although it is unclear how this phenomenon is mediated, it is apparent that IL-2 caused rapid migration of LAK precursor cells to sites inaccessible to peripheral phlebotomy. Similar decreases in LAK precursor cells appeared in the lymphocytes contained within the thoracic duct lymph in patients receiving IL-2 (56).

With continuous administration of recombinant IL-2 at doses greater than 1000 units/kg/hr, circulating Tac-positive cells accounted for as many as a third of all peripheral mononuclear cells (56). Further studies (57) demonstrated that a majority of the Tac-positive cells were $Leu^{2-,3+}$. That these cells indeed expressed messages for the IL-2 receptor was confirmed by use of a cDNA probe for the IL-2 receptor. IL-2 could inhibit anti-Tac binding on these cells, whereas recombinant insulin could not.

Major toxicities occurred with the administration of large doses of recombinant IL-2 (58). Fever and chills developed in every patient receiving

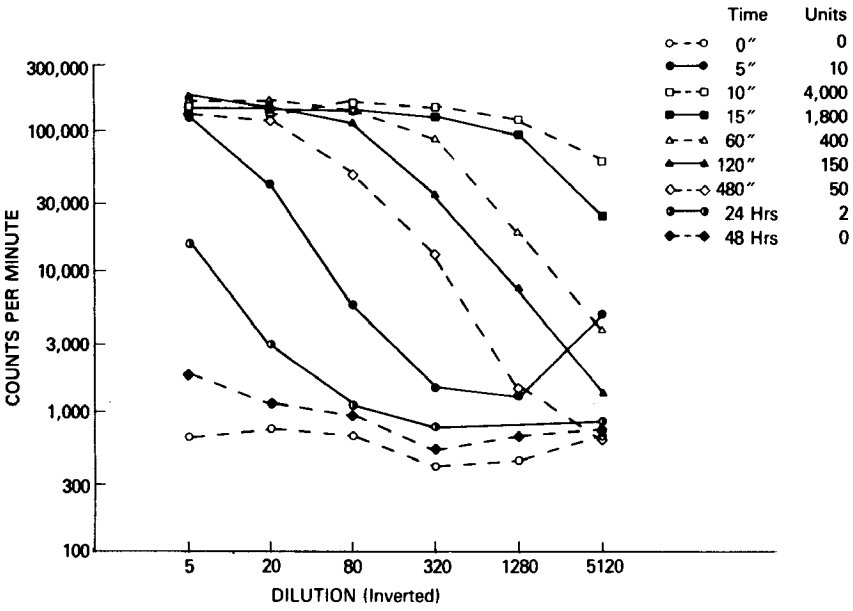

Figure 1 Titration of IL-2 in the serum of a patient receiving recombinant IL-2 by bolus infusion of 10^6 units/kg. Timed serum samples were obtained and tested in a biologic assay with the CTLL murine IL-2 dependent cell line. Low levels of activity could be detected up to 24 hr following this dose.

Table 4 LAK precursors markedly decrease early after IL-2 administration

		Days in culture with IL-2[a]					
		1 day		3–4 days		7 days	
Patient	IV bolus dose of IL-2	Fresh tumor lysis[a]	K562 lysis[a]	Fresh tumor lysis[a]	K562 lysis[a]	Fresh tumor lysis[a]	K562 lysis[a]
1	100,000 U/kg						
	Pre	ND	ND	16.9±3.0	38.3±11.9	31.1±3.7	61.3±11.0
	Post (30″)	ND	ND	−4.6±4.4	3.8±0.5	−10.9±5.3	11.6±1.5
2	300,000 U/kg						
	Pre	ND	ND	36.8±8.0	140.0±12.6	18.0±5.1	51.1±2.0
	Post (4 hr)	ND	ND	1.1±1.0	0.9±3.5	0.8±1.7	−8.5±5.1
3	1,000,000 U/kg						
	Pre	19.9±5.3	49.9±6.1	33.5±3.4	67.1±1.8	57.1±10.2	79.0±5.3
	Post (15″)	7.5±2.1	−6.8±2.2	−2.0±1.6	1.9±3.3	−1.2±0.7	3.5±1.2

[a] % cytotoxicity at 20:1 E:T ratio.

more than 1000 U/kg of IL-2 as a bolus or as an hourly continuous infusion. These symptoms were readily inhibited by the administration of acetaminophen and the prostaglandin inhibitor, indomethicin. Moderate headache, malaise, nausea and vomiting, and diarrhea developed in most patients. One of the most striking toxicities in treated patients was the reproducible weight gain related to marked fluid retention from what was presumed to be a capillary-leak syndrome. Unless patients received exogenous fluids, a syndrome occurred marked by hypotension, tachycardia, and oliguria. Weight gains of as much as 20 kg were noted over a 3-week period. In conjunction with this profound weight gain, other toxicities appeared in some patients; these included confusion, hypoxia related to development of an acute respiratory distress syndrome, hyperbilirubinemia and the development of significant renal dysfunction. A marked eosinophilia developed in most patients, often at levels exceeding those seen in parasitic infections or in the idiopathic eosinophilia syndrome. No apparent toxocity was associated with these high levels of circulating eosinophils, which sometimes amounted to as many as 90% of the circulating white cells. Similar toxicities have been reported by others using IL-2 taken from identical sources (Cetus Corporation) or from other manufacturers (Biogen) (59, 60). All of these side effects were reversible with the discontinuation of IL-2. Though we saw no antitumor responses in patients treated at lower doses of IL-2, we have recently seen significant tumor shrinkage in 3 patients with melanoma receiving 100,000 units/kg of IL-2 3 times a day.

THE ROLE OF INTERLEUKIN-2 IN ADOPTIVE IMMUNOTHERAPY

General Principles of Adoptive Immunotherapy

Experience with a large number of animal tumor models has demonstrated that the systematic transfer of lymphoid cells with specific antitumor reactivity is capable of mediating the regression of established invasive, as well as metastatic, tumors (2–10). Use of carefully selected immunogenic experimental tumor models and the ability to immunize large numbers of normal mice to provide sufficient numbers of immune lymphoid cells for adoptive transfer have been requirements in these studies. Though this immunization approach to the generation of cells used for adoptive transfer is not applicable in humans, these tumor models have established important principles for the application of adoptive immunotherapy in the human.

The most extensively studied tumor model of successful adoptive immunotherapy is the FBL-3 lymphoma in C57BL/6 mice (7, 9), a Friend-

virus-induced leukemia-lymphoma that grows rapidly in irradiated syngeneic C57BL/6 mice but only sporadically in nonirradiated animals. This model has been extensively studied by Fefer, Cheever, and Greenberg (7) and by Eberlein, Rosenberg, and colleagues (9), who established a variety of important principals concerning successful adoptive immunotherapy of this tumor (Table 5). The adoptive transfer of both established local, as well as disseminated, lymphoma can be mediated by the adoptive transfer of either immune-fresh splenocytes, in vitro boosted-immune splenocytes, or immune splenocytes expanded in IL-2 in vitro (9, 49, 51, 61–63). The high degree of immunogenicity of this tumor and its inability to grow in nonirradiated animals, however, raises concern about the applicability of these findings to the treatment of spontaneous human tumors.

A more realistic model for specific adoptive immunotherapy studies in murine models has been developed by Shu & Rosenberg using newly methylcholanthrene-induced sarcomas in early transplant generations (10, 64). These workers have demonstrated that established, growing methylcholanthrene-induced tumors can be made to regress by the systemic adoptive transfer of specifically immune splenocytes. A summary of findings utilizing this tumor model is shown in Table 6.

These tumor models have helped to demonstrate that established tumors can be made to regress by immunologic maneuvers; they also helped to establish several principles, jointly applicable to both model systems, that probably will pertain to the treatment of human tumors by adoptive immunotherapy as well. Specific adoptive immunotherapy appears capable

Table 5 Systemic adoptive immunotherapy of disseminated FBL-3 lymphoma (footpad model) 9, 49, 51, 61–63)

Regression of established local and disseminated tumor can be mediated by the adoptive transfer of:
—immune fresh splenocytes
—in vitro boosted immune splenocytes
—immune lymphoid cells expanded in interleukin-2 (IL-2).
Transfer of both Lyt-1$^+$ and Lyt-2$^+$ lymphoid cells is essential for mediating regression of established tumor.
Host macrophage function plays an essential role in the tumor regression mediated by the adoptive transfer of immune lymphocytes.
When cloned cytolytic and cloned proliferative cell lines reactive specifically with FBL-3 lymphoma were adoptively transferred, only cloned lines with proliferative reactivity could prolong survival of tumor-bearing mice.
Both cytolytic and proliferative clones were Lyt-2$^+$; proliferative responses to FBL-3 were H-2 restricted (KD antigens).
Systemic administration of IL-2 can enhance the efficacy of adoptively transferred murine lymphocytes.

of mediating the regression of established locally invasive and metastatic tumors, though it requires the adoptive transfer of relatively large numbers (approximately 10^8 immune cells to control a 5–10 mm diameter tumor in a mouse). In both model systems the transferred fresh lymphoid cell capable of mediating tumor regression appears to be Thy-1^+ Lyt-1^+2^+ (62). Some degree of in vivo proliferation of these cells appears necessary since the irradiation of cells prior to adoptive transfer abrogates their therapeutic effectiveness (9). As in other tumor models, successful adoptive immunotherapy with these specifically immune cells is dependent on prior immunosuppression of the host using either total body irradiation or treatment with cyclophosphamide (6, 9, 10).

The use of specific adoptive immunotherapy in the human will almost surely involve some aspect of in vitro sensitization to tumor antigens to generate specifically immune cells. Successful adoptive immunotherapy with these stimulated cells is either critically dependent on, or can be substantially enhanced by, the concomitant administration of IL-2 and in vitro sensitized or expanded lymphoid cells (49, 51). The administration of IL-2 appears to have little impact on adoptive immunotherapy utilizing fresh immune cells; however, the in vitro sensitization procedure appears to result in the generation of IL-2 receptors on specifically immune lymphoid cells and the responsiveness of these cells to IL-2. This leads to their proliferation in vivo, which is an important component of their successful therapeutic effect.

The adoptive immunotherapy of human tumors using specifically immune lymphocytes is not yet feasible because of the lack of reliable techniques for the generation of human lymphocytes with specific antitumor reactivity. Should cells with antitumor reactivity become available for use in humans, the simultaneous transfer of IL-2 to sustain the in vivo survival and expansion of these cells will likely be required.

Table 6 Systemic adoptive immunotherapy of established newly induced murine sarcomas (10, 64)

Regression of established intradermal sarcomas can be mediated by the systemic adoptive transfer of specifically immune splenocytes.
Tumor regression mediated by the adoptive transfer of immune splenocytes is tumor-specific and can be mediated only by splenocytes immune to that tumor.
Successful adoptive therapy is dependent on prior immunosuppression of the host (irradiation or cyclophosphamide treatment).
The transferred cell mediating tumor regression is Thy$^+$ Lyt-1^+2^+.
Irradiation of transferred cells abrogates their therapeutic effectiveness.
Adoptive transfer of in vitro boosted immune cells is effective in mediating tumor regression only when Interleukin-2 (IL-2) is administered concommitantly. IL-2 administration does not, however, boost the therapeutic effectiveness of fresh immune cells.

The inability to generate lymphoid cells with specific immune antitumor reactivity has greatly impeded the application of this approach to the treatment of human cancer. However, an unexpected effect of IL-2 in vitro has led to an alternative (12, 13). The use of IL-2-stimulated, lymphokine-activated killer (LAK) cells in immunotherapy has already achieved some success in the treatment of established murine and human tumors (5, 17, 18). Because of the current applicability of this approach, the remainder of this review will deal with recent developments in the use of LAK cells in conjunction with IL-2 cancer immunotherapy.

Lymphokine-Activated Killer Cells

IL-2 acts primarily to stimulate proliferation of T-lymphoid cells bearing the IL-2 receptor. However, in 1980, we observed that the incubation for 3–5 days of either murine or human lymphoid cells in supernatants containing IL-2 was capable of generating lymphoid cells (LAK cells) that could mediate the lysis of fresh tumor target cells, though not of fresh normal cells (12, 13). The most striking feature of this phenomenon is the ability of these LAK cells to lyse fresh, noncultured tumor cell preparations that are natural-killer-cell resistant, either syngeneic or allogeneic, primary or metastatic (14). In this regard these cells are quite different from natural killer cells whose major lytic activity is against carefully selected cultured cell lines, with little reactivity against fresh tumor cell preparations. An example of the generation of murine LAK cells from normal splenocytes is presented in Table 7, and the generation of human LAK cells from normal peripheral blood lymphocytes is shown in Table 8.

The characteristic elements of LAK lysis are clearly exhibited in the murine model. Fresh uncultured cells are incapable of killing a variety of syngeneic fresh target cells despite the presence of natural killer activity in these cells (as shown by their ability to lyse the YAK tumor target line). Culture of fresh splenocytes in complete medium alone does not result in the generation of lytic cells. However (final column in Table 7), the culture of

Table 7 Generation of murine LAK cells

Target	C57BL/6 Splenocyte Effector Cells[a]		
	Fresh (uncultured)	Cultured in medium	Cultured in IL-2
MCA-102 sarcoma	1±1	2±2	53±2
MCA-106 sarcoma	3±1	4±1	53±1
EL-4 lymphoma	−20±2	−18±1	43±2
Normal splenocyte	−12±3	−20±5	8±2
YAC	20±1	8±1	72±1

[a] % lysis (±SEM); 100:1, effector:target (from Reference 16).

fresh lymphocytes in IL-2 is capable of generating cells lytic for these fresh, NK-resistant, tumor cells. Similarly in the human (Table 8), fresh lymphocytes are incapable of lysing either autologous fresh sarcoma cells or a variety of allogeneic cells despite the fact that these cells have natural killer activity, as exemplified by their ability to lyse the NK-susceptible K562 cell line. The incubation of these same lymphoid cells in IL-2 for 3–5 days gives rise to LAK cells capable of lysing these fresh tumor target cells. The LAK cells lyse not only autologous sarcoma but a variety of allogeneic sarcomas and carcinomas as well. Extensive in vitro studies of the characteristics of murine and human LAK cells are summarized in Tables 9 and 10. Several of these findings require emphasis. In the human the precursor of the LAK cell is a non-B, non-T, "null" lymphocyte. Lysis of target cells exhibits no MHC restriction, and a large number of allogeneic tumor preparations can be lysed. Of 41 consecutive preparations of human tumor target cells, 36 were susceptible to significant LAK lysis (Table 11).

LAK cells are also capable of killing cultured normal cells and, in fact, even minor perturbations in the normal cell surface appear to convert normal cells to sensitivity to LAK lysis. Concanavalin-A induced lymphocyte blasts—normal skin fibroblasts—in culture, and even normal cells with cell-surface modifications such as trinitrophenylation appear susceptible to LAK lysis. It is important to emphasize, however, that in the fresh nontransformed state, normal cells are not susceptible to lysis by LAK cells.

Though early experiments were performed using crude supernatants

Table 8 Generation of human LAK cells

	Effector Cell[b]			
	LAK		Fresh PBL	
Target cell	40:1[a]	10:1	60:1	15:1
Autologous sarcoma	76±6	73±1	−4±10	−9±1
Allogeneic sarcoma	88±3	78±2	7±2	−1±1
Colon cancer	62±1	41±3	−16±1	−14±1
Esophageal cancer	78±5	62±2	0±4	−1±1
Adrenal cancer	68±2	41±3	−16±1	−20±2
Pancreas cancer	28±5	17±2	−5±1	−2±1
Normal lymphocyte	4±1	9±6	−9±3	−8±2
K562 cultured cell line	105±6	89±2	46±3	15±1

[a] Effector : target (Reference 65).
[b] % lysis ±1 SEM.

Table 9 Characteristics of murine lymphokine activated killer (LAK) cells

Generated by incubation in interleukin-2 (IL-2).
Peak lysis observed from 3 to 6 days after onset of incubation in IL-2.
Lyse fresh, noncultured, NK resistant, primary and metastatic tumor cells but do not lyse normal cells.
Syngeneic, allogeneic, and exogeneic tumor cells are lysed (murine LAK lyse human tumors and vice versa).
Frequency of LAK precursor lymphocyte is about 1 in 5000 splenocytes as measured in limiting dilution assays.
Precursor of the LAK cell is a non-B, non-T lymphocyte (Thy-1$^-$, Ia$^-$, surface Ig$^-$; thus is a "null" lymphocyte).
Precursor LAK lymphocyte bears asialo-GM1 antigen.
LAK effector cell (resulting from incubation in IL-2) is Thy 1$^+$. Thus LAK cells are probably "primitive" cells in the T-lymphocyte lineage.
LAK cells bear cytolysin granules. Lysis can be inhibited by anti-LFA-1 monoclonal antibody and is partially inhibited by anti-Lyt-2 antibody.

containing IL-2, the availability of highly purified recombinant IL-2 has clearly demonstrated that IL-2 is the sole lymphokine responsible for the generation of LAK cells (24). The availability of large amounts of recombinant IL-2 and the ability to obtain large numbers of lymphocytes by repeated leukophereses in humans provided the opportunity to study the ability of LAK cells with IL-2 to mediate therapeutic effects in humans. Before beginning these efforts, however, a large number of animal studies were performed to define the in vivo effectiveness of LAK cells in mediating the regression of established tumors.

Table 10 Characteristics of human lymphokine activated killer (LAK) cells

LAK cell precursors
Nonadherent
E-rosette negative
OKM-1$^-$
OKT-3$^-$
Leu 1$^-$
Leu 7$^-$
Present in peripheral blood, spleen, lymph nodes, bone marrow, thoracic duct
LAK cell effector
Nonadherent
OKM-1$^-$
OKM-3$^-$
Lyse autologous and allogeneic fresh tumor cells but not fresh normal cells

Table 11 LAK cells kill a variety of fresh human tumor targets

Diagnosis	Number of specimens tested	Number killed by LAK cells (>10% specific lysis)	% Lysis (mean)
Soft tissue sarcoma	20	18	49
Osteosarcoma	5	5	50
Adenocarcinoma			
Colon	7	4	20
Ovary	2	2	23
Pancreas	2	2	24
Lymphoma	3	3	41
Esophageal carcinoma	1	1	78
Adrenal carcinoma	1	1	68
Total	41	36	

(From Reference 65.)

The Role of LAK Cells in Cancer Immunotherapy

Many studies have now demonstrated that the adoptive transfer of LAK cells in conjunction with recombinant IL-2 can mediate the regression of established pulmonary and hepatic metastases from a variety of murine tumors in several mouse strains (17, 18, 22, 48, 66). In these experiments, therapy was begun from 3 to 10 days after tumor cell injection, at a time when established metastases were evident. The efficacy of therapy was evaluated by counting in a coded fashion the number of either pulmonary or hepatic metastases at approximately 2 weeks after tumor cell injection, or by evaluating survival of mice. A typical experiment is shown in Table 12.

Table 12 Immunotherapeutic effect of LAK cells and recombinant interleukin-2 on established pulmonary sarcoma metastases

	Number of metastases (mean) at day 13[a]	
Cells[b]	HBSS	RIL-2
None	228	152
Cultured splenocytes	183	156
Fresh splenocytes	214	191
LAK	200	20[c]

[a] The number of MCA sarcoma cells injected iv was 3×10^5; each group contained 6 mice. RIL-2 (15,000 U/injection) or HBSS was injected ip approximately every 8 hr from days 3–8 after tumor injection.

[b] 1×10^8 LAK cells, fresh normal splenocytes, or normal splenocytes cultured for 3 days without RIL-2 were injected iv on days 3 and 6 after tumor injection.

[c] LAK + RIL-2 vs RIL-2 alone; $p < 0.01$. (After Reference 66.)

Cells from an early generation, methylcholanthrene-induced sarcoma were injected intravenously, and 3 and 6 days later 10^8 syngeneic LAK cells' normal splenocytes, or splenocytes cultured in vitro in the absence of IL-2, were injected into these mice. One half of mice in each group also received the intraperitoneal injection of 15,000 units of IL-2 every 8 hours from 3 to 8 days after tumor injection. Fresh splenocytes, or splenocytes cultured in the absence of IL-2, had no impact on the number of metastases. IL-2 alone at this dose, or LAK cells alone, had little or no impact on the number of metastases. However, combination therapy, with LAK cells and with recombinant IL-2, reduced by greater than 90% the number of pulmonary metastases in these mice (from 228 metastases to 20 metastases, $p < 0.001$). This ability to reduce pulmonary metastases was highly reproducible. Experiments demonstrated that the infusion of both LAK cells and IL-2 was required for the maximal reduction of pulmonary metastases. Though high doses of IL-2 alone are capable of reducing pulmonary metastases (see earlier section), at the low doses of IL-2 used in these experiments, the administration of LAK cells was required. At any dose of IL-2 the combination of LAK cells and IL-2 was more effective in reducing metastases than was therapy with IL-2 alone.

A typical experiment demonstrating the effectiveness of LAK and IL-2 therapy in the treatment of hepatic metastases is shown in Table 13. This experiment again demonstrates that the combination of LAK cells and IL-2 is essential to mediate antitumor effects. It is important to emphasize that successful adoptive therapy with LAK cells and IL-2 can be achieved using

Table 13 Effects of LAK cells versus fresh cells and cultured cells with and without RIL-2 on the reduction of day-3 established liver metastases

Group	Treatment		Number of metastases (mean)[c]
	Cells[a]	RIL-2[b]	
A	None	−	246
B	None	+	53
C	LAK	−	193
D	LAK	+	1
E	Cultured splenocyte	−	250
F	Cultured splenocyte	+	50
G	Fresh splenocyte	−	245
H	Fresh splenocyte	+	60

[a] 1×10^8 LAK cells, fresh normal splenocytes, or splenocytes cultured for 3 days in complete media without IL-2 were given iv on days 3 and 6 after tumor injection.

[b] Mice received 7500 units per injection of RIL-2 from days 3–10 after tumor injection.

[c] Statistical significance of differences: A vs B, $p < 0.005$; C, $p < 0.05$; D, $p < 0.005$; E, nonsignificant; F, $p < 0.005$; G, nonsignificant; H, $p < 0.005$; B vs D, $p < 0.005$; F, nonsignificant; H, nonsignificant. (Reference 48.)

nonimmunogenic tumors. A characteristic experiment demonstrating this point is shown in Figure 2. The MCA-102 sarcoma utilized in this experiment is nonimmunogenic, and all attempts to immunize mice against this tumor using a variety of techniques have failed. Whereas high doses of IL-2 alone are capable of providing a minor reduction in the number of metastases seen in this tumor model, the reduction in metastases seen with the combination therapy of LAK cells and IL-2 is far greater.

A large number of experiments have now been performed analyzing the parameters important for the successful immunotherapy of established murine lung and liver metastases with LAK cells and IL-2. The findings of these experiments are summarized in Table 14 (17, 18, 22, 48, 66). Several points require emphasis. Therapy with LAK cells and IL-2 is successful against a variety of immunogenic and nonimmunogenic sarcomas and carcinomas. The effective cell in vivo is derived from a non-T-cell (Thy-1$^-$) precursor that develops into a Thy-1$^+$ cell in vitro under the influence of IL-2 stimulation. LAK cells effective in adoptive immunotherapy can be generated from tumor-bearing animals as well as from allogeneic normal mice. In contrast to the use of specific immune cells, immunotherapy with

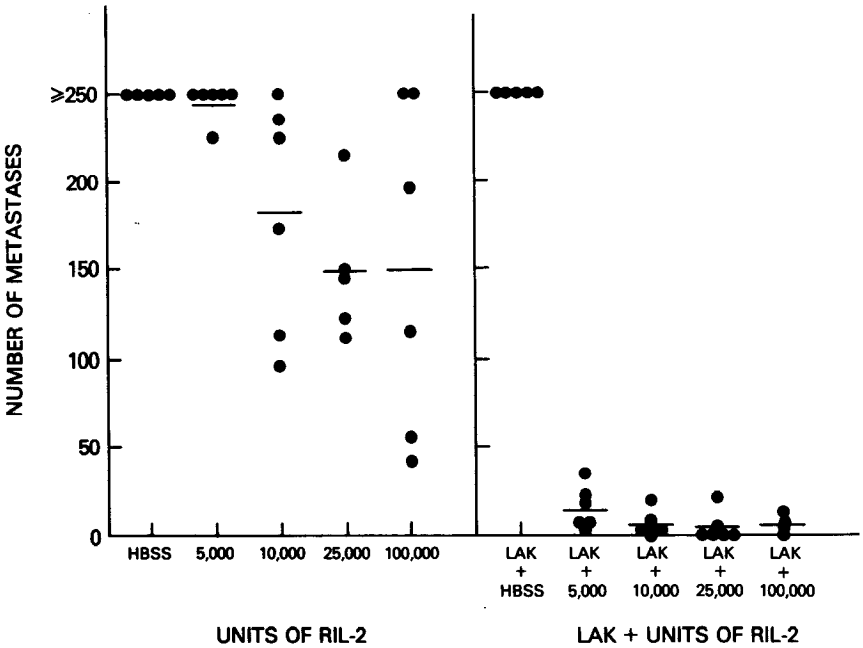

Figure 2 Effect of RIL-2 and LAK cells plus RIL-2 on therapy of MCA liver metastases Reduction of liver metastases in mice bearing the MCA-102 nonimmunogenic sarcoma treated with either IL-2 alone or LAK cells plus IL-2.

LAK cells and IL-2 is effective in the normal host as well as in hosts whose immune system has been suppressed by total body irradiation or treatment with cyclophosphamide. Thus, immune depression of the host is not required to achieve successful LAK therapy. In all of the animal model systems studied, the more LAK cells transferred and the larger the doses of IL-2 administered, the greater the therapeutic effects against established tumors.

The mechanism of the therapeutic effect of LAK cells and IL-2 is under intensive investigation. It appears clear in murine models that the adoptively transferred LAK cells proliferate in vivo in a variety of host tissues under the stimulation of IL-2 (67). Using the adoptive transfer of congenic Thy-1.2 LAK cells into Thy-1.1 host mice, the proliferating cells in mouse organs were shown to be of donor rather than of host phenotype. Further, following the injection of LAK cells plus IL-2, it was possible to recover expanded numbers of lymphocytes with LAK activity against fresh murine tumor from the lung or liver itself. It thus appears that LAK cells with antitumor reactivity proliferate in vivo under the influence of IL-2 and that these expanded cell populations maintain their lytic activity. These expanding lymphocytes appear capable of mediating antitumor effects in

Table 14 Characteristics of the immunotherapy of established murine lung and liver metastases with LAK cells and IL-2 (17, 18, 22, 48, 66)

Established lung and liver metastases can be inhibited by treatment with LAK cells and IL-2. Both LAK cells and IL-2 are required, and either administered alone is less effective.
A direct relationship exists between the number of LAK cells administered and the therapeutic effect.
A direct relationship exists between the amount of IL-2 administered and the therapeutic effect.
Incubation of splenocytes for 3 days appears optimal for the generation of LAK cells effective in vivo.
Immunotherapy with LAK cells and IL-2 is effective in hosts suppressed by total body irradiation or treatment with cyclophosphamide. Therapy is also effective in "B" mice (thymectomized, lethally irradiated, reconstituted with T-cell-depleted bone marrow).
Allogeneic LAK cells are effective in conjunction with IL-2.
Irradiated LAK cells (3000 rads) are less effective than nonirradiated cells.
LAK cells effective in immunotherapy can be generated from the splenocytes of tumor bearing mice.
Successful therapy of immunogenic and nonimmunogenic sarcomas, melanoma, and a colon adenocarcinoma has been demonstrated.
The precursor of the LAK cells effective in vivo is a non-T cell (Thy 1^-).
Metastases that persist after in vivo therapy with LAK cells plus IL-2 are sensitive to LAK cell lysis both in vitro and in subsequent in vivo experiments.
Administration of IL-2 leads to in vivo proliferation of transferred LAK cells.

vivo. Though diffuse lymphoid infiltrates appear in most mouse organs and presumably result in toxic side effects, these cells are exquisitely sensitive to the presence of IL-2. When IL-2 administration is discontinued, these lymphoid infiltrates resolve over the course of several days.

Therapy of Cancer in Humans Utilizing LAK Cells and IL-2

The results of therapy with LAK cells plus IL-2 in experimental tumor models predicted that maximal effects from using this approach for the treatment of human cancer would be expected when therapy involved both LAK cells and IL-2. The dose of IL-2 alone required to mediate consistent antitumor effects in murine models was 2.5×10^6 units of IL-2/kg administered every 8 hours. This dose is approximately 25 times higher than that tolerated by humans on a per kilogram basis. In contrast, 2.5×10^5 U/kg of IL-2 was capable of supporting the antitumor effects of LAK cells in murine models.

Early efforts to apply the use of LAK cells and IL-2 to the treatment of human malignancy proceeded in three stages. First it was necessary to demonstrate that recombinant IL-2 and activated killer cells could be given separately, with safety, to humans. This was a prerequisite to the combination of these treatments in patients with advanced cancer. The ability to generate LAK cells in numbers sufficient for transfer to patients with cancer was dependent upon large amounts of recombinant IL-2, which first became available in 1984 (23, 24). However, between 1980 and 1982, a variety of methods for activating human lymphoid cells to generate cells that were active in lysing fresh human tumor were described by Rosenberg and colleagues (68–71). These techniques included the stimulation of human peripheral-blood lymphocytes with lectins such as phytohemagglutinin (phytohemagglutinin-activated killer cells—PAK) or by the use of pooled allosensitization as previously described by Zarling & Bach. Using human activated–killer cells (either PAK or LAK cells) for the treatment of 27 patients with advanced cancer, early clinical trials were conducted between 1982 and 1984 (72, 73). Though no antitumor effects were seen in any patient, these clinical protocols demonstrated that relatively large numbers (nearly 2×10^{11}) of activated killer cells could be administered to humans with minimal toxicity. The major toxicities associated with the administration of activated killer cells alone were transient fever and chills, and these could easily be eliminated by the use of appropriate medications. Pulmonary diffusion capacity decreased transiently, then returned to normal hours after cell administration. Cells for these studies were obtained by repeated leukophereses of these cancer patients, and this procedure was also well tolerated.

Following the repeated infusion of activated killer cells from successive leukophereses, the appearance of cells lytic against autologous tumor was measured in the peripheral blood of these patients (72). In addition the traffic of these activated killer cells was evaluated utilizing Indium^{111}-labeled cells. Following the first infusion of Indium^{111}-labeled PAK cells, these cells were promptly cleared by the liver and spleen. Following the ninth infusion of PAK cells, however, excretion of these cells was significantly delayed, and increased numbers of infused cells were found in the lung.

In December 1984, the first clinical trials were initiated using LAK cells plus IL-2 for the treatment of patients with a variety of advanced malignancies. An important part of the evolution of this clinical protocol was the performance of the cell harvests at a time after the IL-2 administration had stopped. This was essential because, as our findings described previously, LAK precursors disappeared from the circulation following IL-2 administration.

Of the 22 evaluable patients with advanced cancer treated with LAK cells plus IL-2, 9 patients have shown an objective regression of metastatic cancer. These responses have occurred in four different histologic types of cancer including colon cancer, melanoma, renal cell cancer, and a primary lung adenocarcinoma. Metastases in the lung, subcutaneous tissues, and liver have regressed during these therapies.

One patient has experienced a complete regression of all metastatic melanoma that has lasted twelve months following the completion of therapy. This patient was a 29-year-old woman with metastatic melanoma who had previously failed surgical resection and therapy with interferon. She was treated with a total of 4.2×10^{10} LAK cells obtained from 9 successive leukophereses. Concomitantly, she received 47 doses of IL-2 comprising a total of 7.9×10^5 total units of recombinant IL-2 per kg. This patient had multiple subcutaneous melanoma metastases in the arm, thigh, back, and buttock. All lesions began to regress following the completion of therapy with LAK cells and IL-2, and by three months after the completion of therapy, all lesions had disappeared.

The use of LAK cells plus recombinant IL-2 appears to hold some promise for the treatment of human malignancy. Principals established from animal models appear to be applicable to the treatment of humans with malignancy. Currently, efforts are underway to test further the use of this approach for the treatment of cancer in humans. The toxicity associated with the administration of IL-2 and the number of cells that can be obtained by repeated leukophereses represent limits to the application of this therapy. Means to overcome these limitations are currently under active investigation.

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CONTENTS