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SHEER LUCK MADE ME AN IMMUNOLOGIST

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A Head Start

The luck began when I was four years old and my younger brother contracted typhoid fever from a carrier in the local dairy. It was not a lucky event for him, of course; he became desperately ill and was not expected to live, so my parents spent months in the hospital by his side.

It turned out to be lucky for me, however, because I was farmed out to the next-door neighbors who had two preteen daughters. For some reason, the girls decided to undertake my education. They knew nothing of the techniques, neither the do's nor the don'ts; they just taught me to read simple words and sentences and to count. Proud of their handiwork, they took me to school and had me show off my accomplishments. This was very heady stuff for a four year old who was feeling, rightly or not, somewhat left out by her parents.

My brother finally recovered from typhoid fever, but his immune system was so depleted that he promptly succumbed to every known childhood disease. As a consequence, I was quarantined at home for a year, and my martinet father took over my education. When I finally was allowed to go to school, it was clear that the tutoring by the girls and my father had put me ahead of my contemporaries, and this assurance served to support my conviction that "studies" was the one thing I was good at.

Upward Bound

My second piece of good luck was provided by three Jewish boys who were my main friends during high school, particularly during the last two years. I emphasize the word "friends" because in those days religious differences meant that I could never for a moment be considered a "girl friend." We were simply a gang who did things together, and since they came from a more cultured

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and ambitious tradition than mine, I was challenged to go along and keep up with them both outside and inside school. Outside of school during the 1930s depression, we went to WPA theater productions such as *One Third of a Nation* and *Murder in the Cathedral*; we sat at the top of the Metropolitan Opera House to hear Kirsten Flagstad and Lauritz Melchior sing *Tristan and Isolde*; we visited the downtown fish market at dawn and browsed through Chinatown. Inside school, when my friends chose to take a physics course, I went along and, much as I disliked it, learned to wire a light box and measure gas pressure. When they got A's in American history, I had to do as well. And when they picked up a three-foot constrictor black snake in biology class, I followed suit. And then I had to eat the canned rattlesnake meat that was the prize for the only girl who dared to touch the reptile. Thus, I was not only intellectually stimulated during my teen years, but I also escaped the usual pressures to be a "girlie."

Start of a Research Career

I was a sophomore chemistry major at Vassar, bored with memorizing organic reactions, when I was saved by a third stroke of luck. The College hired a PhD fresh from Yale to teach bacteriology and, on the side, a little immunology. The young professor, Catherine Dean, was such a good and enthusiastic teacher that within her first year she collected twelve majors from my class alone—a number that surpassed the usual total of science majors at the College. It was her introduction to the new science of immunology that provided me with a research career for a lifetime. Only a year before, Tiselius and Kabat had finally identified serum antibodies as members of the γ-globulin fraction, and one of the critical questions that arose from these studies was whether the various serum antibody activities—agglutination, precipitation, neutralization, complement-fixation—were carried out by different immunoglobulin molecules or were different manifestations of the same molecule, the so-called Unitarian Hypothesis.

I was much more intrigued by the ability of immunoglobulin molecules to recognize and eliminate an almost infinite number of foreign invaders. I read Karl Landsteiner's *The Specificity of Serological Reactions*, (Charles C. Thomas 1936), which had been published just four years before, and I was determined to try my hand at resolving the basis for the diversity and specificity of antibody reactions. How fortunate I was at the age of nineteen to be able to select a research area that, despite a few dips here and there, has grown and developed over the years and is even more fascinating and important today than it was when I began.

Graduate Reinforcement

My choice of graduate school was based on hard cash: Having spent my life in the East, I wanted to go as far West as my limited amount of money would

allow. The University of Chicago won out as the best institution with the lowest traveling costs, $16 for an overnight coach ticket from New York. Little did I realize that the University had become a hub of wartime research, not only for the development of the atom bomb and various explosives, but also for the control of infectious diseases among the troops. But being broke and having to work my way through graduate school turned out to be the source of great luck because it gave me the opportunity to work on two such projects. Each experience emphasized the need to understand the fundamental mechanisms of immunity, and both reinforced my desire to participate in such research.

The aim of the first project was to develop a vaccine that protected troops in the Far East against cholera. Since the infection is confined to the lumen of the gut, parenterally administered vaccines were of little efficacy. Our studies used an experimental disease in guinea pigs to show that oral administration of a nonlethal dose of vibrios prevented the disease, and the protection was associated with the secretion into the gut of antibody, politely called copro- rather than fecal antibody. These findings provided an important precedent for the subsequent development of the oral polio vaccine and left me with a lifelong interest in antibody secretion into the body cavities.

In the second project, the Commission on Airborne Diseases was charged with reducing the incidence of respiratory disease during Army basic training. Streptococcal infections were particularly prevalent among recruits from rural areas; they had little previous exposure to the causative organism and often developed serious autoimmune complications, such as rheumatic fever and glomerulonephritis. In this case, the problem was solved not by immunological approaches but by controlling transmission of the pathogens. Epidemiological studies showed that the incidence of disease could be significantly reduced by measures that reduced the number of airborne pathogens in the barracks sleeping quarters—measures such as spraying a thin layer of oil droplets on blankets and clothing to prevent the release of bacteria during bedmaking.

Career Crisis

Perhaps the greatest luck of all was the support and advice my husband gave at the end of our postdoctoral training at Harvard. Up to that time, I had proceeded along the usual route, marriage, graduate school, two children, postdoctoral studies, and had blithely assumed the usual outcome, a full-time academic career. An unexpected complication arose, however; namely, the arrival of twins, which meant that our family suddenly consisted of four children under the age of five. Reality struck as I had to face how I could do both—be a good mother and a good immunologist. When I told my husband of my decision to become a full-time mother, he would have none of it and instead suggested part-

time work as a solution. He overrode my objections with two arguments; first, that most tenure-track scientists did research only part-time because of teaching and administrative duties, and second, that a half-time research adjunct could remain competitive by being creative and undertaking high-risk projects that a tenure-track scientist could less afford to do.

I followed my husband's advice for twenty years until our youngest son finished high school. It meant that I had a great time doing "crazy" research and did not suffer severe guilt pangs about the children.

A Paradigm for Science Education

Many of the chance events that shaped my career as an immunologist are now the basis of educational programs aimed at generating an interest in learning. Thus, preschool tutoring, such as I had, has been employed by Head Start to remedy the backgrounds of children from disadvantaged homes. The program has an impressive record of turning out children who perform better on entering school and thus like the learning process. Maintaining that performance and liking of learning during the teen years, however, requires programs that can successfully counteract the anti-intellectualism inherent in some social traditions and teenage peer groups. My father was a good example of the traditional problem. His ambition was for me to be a "lady," which translated into taking courses that aimed to produce a good hostess and a good housekeeper rather than a good physicist or a good mathematician. He didn't approve of my Jewish friends, but fortunately he could not override the stimulation and security of their friendship. Programs such as Upward Bound are providing support to high schoolers equivalent to that provided by my Jewish friends. Their aim is to combat not only social pressures but also peer pressures that place the highest values on being a cheerleader, or starring on the football team, or wearing expensive clothes. Organizations such as the National Science Foundation are trying to create rewards for scientific achievement that can compete with the publicity given high school sporting events or prom queens. The best science students in each state and his/her high school teacher meet in Washington and have their pictures taken with "big shots" like the President's Science Adviser or the Director of National Science Foundation.

"Hands-on" experience is a powerful career attractant. A research project in my senior year at Vassar and the subsequent wartime projects completely destroyed any notion I had of alternative careers, even medicine. The research bug had bitten! And my bite has been duplicated hundreds, thousands of times. The Oberlin Report clearly documented that it is an undergraduate research project conducted in close contact with a professor that is responsible for the high turnout of scientists by the small liberal arts colleges and for the high rate of success among those scientists. As a result, "hands-on" laboratory work

has been adopted as a lure by many public and private organizations to interest minorities and women in scientific careers. The MARC program of the National Institutes of Health and the undergraduate programs sponsored by the Howard Hughes Medical Institute are good examples of how effective the hands-on approach can be.

Where Is Creativity?

One of the elements, perhaps the most important one, that shaped my career as an immunologist has, however, fallen through the cracks of most science education programs. That is an emphasis on creativity. At the beginning of my senior year at Vassar, Catherine Dean led the bacteriology majors to a room in the basement and said, "Here is a centrifuge, a pH meter, an incubator, media on the shelf, and various bacterial strains in the icebox. Dream up your research project!" We did, because senior research was a requirement for graduation. In the graduate program at the University of Chicago, students (that included me) were expected to develop a thesis proposal and then sell the project to the appropriate professor in the Department. In the postdoctorate scene at Harvard, appointments could be made to do independent work provided that the proposed work passed faculty review. I took full advantage of the opportunity. The emphasis on originality in these programs reflected the notion that students could be trained to think creatively and that such training was critical to their success in their subsequent careers. This notion was amply supported in my own case because the training enabled me to follow my husband's advice and to remain competitive by concentrating on more "far out" research ideas.

Many of the devices used in the past to foster creativity, e.g., graduate students designing their own thesis projects, have been discarded. They became impractical as the biological sciences grew more sophisticated, more team-oriented, and more competitive. Unfortunately, the emphasis on training in creative thinking has disappeared along with the devices. The de-emphasis has occurred as young scientists have a harder and harder time creating research niches to establish themselves, as study sections award their limited amounts of money to safe rather than innovative proposals, as the number of scientists being trained is reduced without any criteria for selecting those with the most original minds. The de-emphasis has also occurred at a time when creative thinking is needed for young scientists to tackle socio-scientific problems such as the dealing with the question of a second job for a spouse or significant other, the division of time and effort between research and parenting, etc. In my opinion, the immunological community would do well to focus on such issues and devise techniques for encouraging creative thinking that are applicable to the current state of the science world and its teaching.

Bad Luck

At this point in my recital, you may ask whether there were any unlucky events that affected my career. Of course, there were. To give a few examples: A professor at Chicago gave me a foretaste of things to come as I was going off to Harvard for my postdoctorate. He said that I had been an excellent graduate student, but because I was a woman, I should not entertain any hopes of being hired by the faculty of the Department of Bacteriology and Parasitology. At another institution, the head of the department proclaimed he would never hire the wife of anyone, not even the janitor, so my husband and I had to look elsewhere for the second job. Early in my career, one or two immunologists would get up after each of my research presentations and say they couldn't reproduce the data. Although their criticisms were never documented, the constant voicing of doubts had the effect of delaying recognition of my work.

To be fair, there were also many helping hands along the way. The faculty at Chicago sponsored me for membership in the American Association of Immunologists before such well-deserving male peers as David Talmage, Maurice Hilleman, and Riley Hauswright. Dr. Howard Mueller, head of the Harvard Department of Bacteriology, went into the shop and personally made 24 guinea pig metabolism cages that I could not afford to purchase on my fellowship. Dr. Wendell Stanley, head of the Virus Laboratory at Berkeley, bought some $25,000 worth of equipment for my research without any guarantee that I would get a grant and pay him back. I suppose the unkindness and kindness come out about even.

As I look back, I think my one significant piece of bad luck may have been not playing a team sport. Recent analyses have indicated that women scientists tend to be loners who do not belong to a network of collegial associates. Typically they run small laboratory groups and are relatively unaggressive about promoting their research accomplishments. These characteristics contrast sharply with those of the average male colleague, and the question is, why? A number of explanations have been offered: differences in mentoring, in standards for masculine and feminine conduct, etc. One of the most intriguing is the difference in athletic experiences. Most men have participated in some form of team sport throughout their education, whereas most women have not. Moreover, men continue to participate in their adult years; the male graduate students, postdoctorates, and able faculty from our immunology floor still get together for a basketball game using a net rigged up outside the laboratory building. In these team sports, the players get the opportunity to practice competitiveness; they learn how to develop winning strategies and, at the same time, how to work cooperatively and form successful liaisons—very valuable lessons for any

subsequent competitive endeavors. The idea of a contribution from team sports came much too late to affect the education of my three daughters. However, all seven of my granddaughters, ranging in age from five to eighteen, have been or are currently playing on soccer teams! We'll see what happens.

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ALTERED PEPTIDE LIGAND–INDUCED PARTIAL T CELL ACTIVATION: Molecular Mechanisms and Role in T Cell Biology

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ABSTRACT

The elucidation of the phenomena of T cell antagonism and partial activation by altered peptide ligands has necessitated a revision in the traditional concepts of TCR recognition of antigen and subsequent signal transduction. Whereas previous models supported a single ligand specificity for any particular T cell, many studies using analogs of immunogenic peptides have now demonstrated a flexibility in this recognition. Moreover, interaction with such altered peptide ligands can result in dramatically different phenotypes of the T cells, ranging from inducing selective stimulatory functions to completely turning off their functional capacity. Investigations of the biochemical basis leading to these phenotypes have shown that altered peptide ligands can induce a qualitatively different pattern of signal transduction events than does any concentration of the native ligand. Such observations imply that several signaling modules are directly linked to the TCR/CD3 complex and that they can be dissociated from each other as a direct result of the nature of the ligand bound. Interestingly, many in vivo models of T cell activation are compatible with a selective signaling model, and several studies have shown that peptide analogs can play a role in various T cell biologic phenomena. These data strongly suggest that naturally occurring altered peptide ligands for any TCR exist in the repertoire of self-peptides or, in nature, derived from pathogens, and recent reports provide compelling evidence that this

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is indeed the case. The concept of altered peptide ligands, their effects on T cell signaling, the hypothesized mechanisms by which they exert their effects, and their possible roles in shaping the T cell immune response are the scope of this review.

THE CONCEPT OF ALTERED PEPTIDE LIGANDS

Definition of APL

The recognition by T cells of short, linear peptides bound to MHC molecules has been well established (1, 2). In addition, the accessibility of only three to five amino acid side chains of a peptide to the TCR supported the theory that the specificity of TCR recognition is conferred by only a few peptide residues (1, 3, 4). Many recent investigations have focused on the nature of these TCR contact sites (5–7), and observations from these have revealed a previously unforeseen layer of antigen fine specificity, whereby such peptide residues display a hierarchy of interaction and contribution to the resulting signal transduction processes in the T cells.

Series of peptide analogs were generated in a variety of antigen systems by introducing single amino acid substitutions. These analogs were then tested for their capacity to stimulate a variety of T cell responses in panels of T cells specific for the same ligand. From such analyses, several analogs were identified that could stimulate a subset of the normal activation events in the T cells, and the results suggested that some of the interactions with the TCR are more critical than others. Information obtained from such studies led us to assign the labels “primary” or “secondary” TCR interaction sites for the TCR contact sites of a stimulatory peptide (7). The primary contact site was defined as the amino acid focused on most intently by all T cells in a population specific for the same antigen, and this amino acid appeared to be the most critical residue of the determinant to the TCR binding. Secondary residues were defined as the other TCR contact sites, which were unique to each TCR in the population, and they were assumed to play a lesser role in the overall interaction between TCR and ligand. We subsequently defined the term “altered peptide ligand” (APL) to describe analogs of immunogenic peptides in which the TCR contact sites have been manipulated (7). While these peptides do not stimulate T cell clonal proliferation, they nevertheless have the capacity to activate some TCR-mediated effector functions. Other peptide analogs do not stimulate any detectable function from the T cells and are simply termed peptide analogs.

APL and Partial Activation

The initial observation that T cell stimulatory events could be dissected was made by our laboratory using a Th2 clone specific for the Hb(64–76) peptide bound to an I-E^k molecule (8). Whereas the original stimulatory peptide could trigger proliferation, IL-4 production, and the provision of B cell help from this T cell clone over a similar dose range, an analog containing a conservative amino acid substitution effectively stimulated IL-4 production and induction of B cell helper function, but not T cell proliferation.

Subsequent to the initial demonstration of partial T cell activation, the phenomenon of analog-induced T cell antagonism was described by Sette and colleagues (9). This phenomenon was shown by the simultaneous presentation of agonist and nonstimulatory peptides to T cells; the antagonism involved a mechanism whereby the analog specifically downmodulated the agonist-induced response. These data are discussed at length in a later section of this chapter. Partial T cell activation was next shown in Th1 cells, indicating that it was a global phenomenon and not just peculiar to the original Th2 clone in which it was defined. In Th1 cells, APL stimulated cytolytic activity, cell volume increase, and upregulation of cell surface IL-2 receptor levels, but APL did not induce proliferation, cytokine production, or phosphoinositol hydrolysis (10, 11). A report from another group later confirmed the partial activation phenotype in a murine alloantigen system (12). Because addition of peptide inhibited some TCR-mediated events (IL-2 production) but not others (IL-3 production, IL-2 receptor α expression and cell enlargement), the investigators concluded that this was a demonstration of partial T cell activation. Together, the above data using partially activating APL suggested that selective T cell signaling events were being stimulated in the absence of others.

APL and Anergy Induction

An analysis of the consequences of delivering a partial signal by APL revealed that the T cells had been rendered functionally anergic, subsequently unable to respond to stimulatory ligand (11, 13). This phenomenon was dependent on the partial signal, since neither a stimulatory dose of agonist ligand nor analogs that did not partially activate the T cells could induce the long-term anergic phenotype (11). These observations were consistent with earlier studies demonstrating that TCR engagement in the absence of T cell proliferation leads to T cell anergy (14–16). However, several important differences in the two systems were noted. While the classical studies used stimulatory ligand and costimulation-deficient antigen presenting cells (APC) to anergize T cells (14), APL-induced anergy occurred in the presence of live, functional APC (11). Moreover, addition of exogenous costimulation was unable to rescue the T cells

from APL-induced anergy, indicating that other components of the T cell signaling cascade were missing. Finally, while antigenic ligand and nonfunctional APC stimulated some T cell cytokine production and phosphoinositol hydrolysis, neither of these activation events could be detected upon TCR engagement of APL and live APC in Th1 cells (17, 18). Together, these observations suggested that the signaling component missing in APL stimulation of T cells did not involve costimulation and was likely a very early membrane proximal event.

APL and T Cell Antagonism

T cell antagonism was first defined as a specific and dose-dependent downmodulation of agonist-induced T cell proliferation when both agonist and APL were presented to the T cells simultaneously, and such antagonism was hypothesized to be due to competition for TCR sites (9). However, since the ligand for TCR is made up of two components, competition between the two peptides for MHC binding sites had to be eliminated as a possible explanation for the observed results. This issue was addressed in several ways. First, APC were prepulsed with stimulatory ligand, at a dose that would occupy only a fraction of the available MHC binding sites. After washing excess peptide away, the APL was then added together with the T cells, and the T cell response assessed. Second, irrelevant competitor peptide, with similar binding affinity for the MHC molecules, could not diminish the T cell response to agonist (9). Finally, APL could antagonize a superantigen-mediated T cell response, confirming that MHC competition was not involved, since superantigen and peptide antigen bind independent sites on MHC molecules (19). APL-induced TCR antagonism has now been reported using a variety of antigen systems, in both $CD4^+$ and $CD8^+$ T cells, and the antagonism affects several cell functions, including cytokine production, cytolysis, serine esterase release, and phosphoinositol hydrolysis (12, 20–22).

Relationship Between Partially Activating and Antagonist APL

Initial studies of T cell antagonism showed that the antagonistic peptides were unable to deliver any identifiable signal to the T cells, including Ca^{++} flux, or cytokine production or induction of T cell anergy (9). The identification of both partial agonist and antagonist APL in the hemoglobin system provided us the unique opportunity to study the relationship between the two (8, 10, 11, 19). By introducing many different residues at a particular TCR contact site, we were able to correlate structural modification with stimulatory ability of the analogs. We found that analog peptides containing very conservative substitutions at defined secondary TCR interaction sites retained the partial activation phenotype, in addition to being efficient antagonists (23; J Sloan-Lancaster, BD Evavold, unpublished observations). As the nature of the substituted amino acid

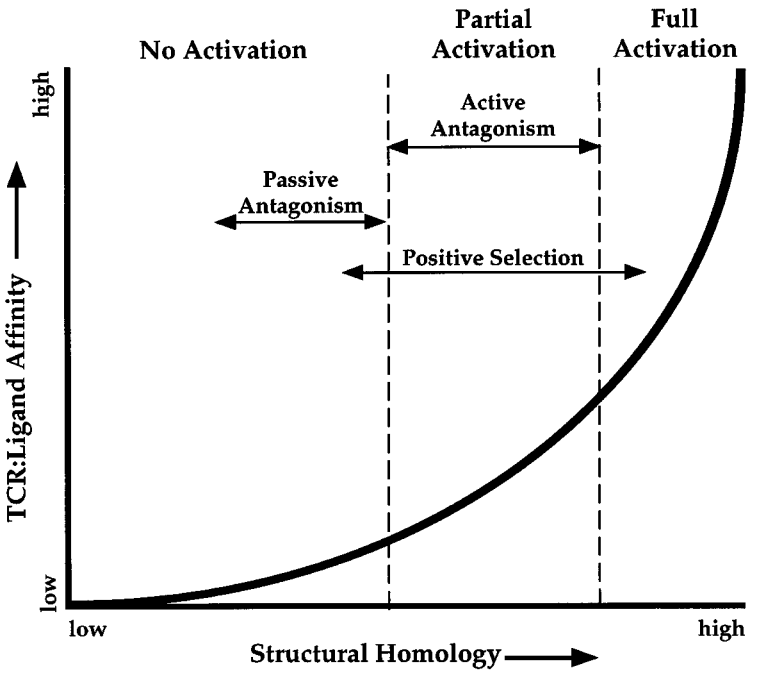

Figure 1 Relationship between antagonistic and partially activating APL. Peptide analogs fall into a spectrum of stimulatory categories related to their structural homology with agonist ligand, which may be due to increasing affinity for the TCR. Thus, partially activating APL are a subset of the antagonists. Antagonism may therefore occur by two distinct mechanisms. We propose that passive antagonism is a surface phenomenon involving direct competition for TCR sites, while active antagonism requires inhibitory signaling events, which are dominant over agonist-induced signals. Analogs able to induce positive selection are positioned based on the available data.

deviated more from the original residue, the partial activation phenotype diminished while the antagonistic function was retained. Eventually the antagonism was also lost, with the analog displaying a null phenotype (23). Interestingly, amino acid substitution at the defined primary TCR contact site, even conservatively, completely abolished all detectable activity of the peptide in all T cells tested (7; J Sloan-Lancaster, BD Evavold, unpublished observations).

The above data are consistent with a threshold-of-activation model for T cell signaling, as shown schematically in Figure 1. Successful stimulation of individual T cell functions seems to depend on activation reaching a certain critical level within the cell, with triggering of each function requiring a particular threshold to be achieved. The capacity of an extracellular stimulus to trigger

activation to different thresholds is likely dictated by the amount of TCR cross-linking induced and the affinity of the ligand for the TCR. Thus, as a peptide analog becomes more structurally homologous to the agonist ligand, activation to higher thresholds will be accomplished, and more effector functions will be stimulated. These can range from antagonism alone to several partial activation phenotypes, depending upon the threshold level reached by the particular peptide analog. Consistent with this, partially activating APL appear to be a subset of the antagonists (7, 23).

Since both pure antagonists and partially activating APL can antagonize a T cell response, the question arises whether they do so by the same or independent mechanisms. We favor the latter choice and propose that two types of APL-induced T cell antagonism exist (Figure 1). Passive antagonism appears to occur without any T cell signaling events and may simply be a surface phenomenon involving direct competition for TCR sites, resulting in dilution of the amount of agonist ligand in a T cell contact cap. This seems to be the less efficient mechanism and should require high concentrations of analog peptide to induce significant inhibition. Many antagonists reported in the literature could fall into this category (9, 20, 22). Active antagonism, in contrast, seems to require an APL to induce some biochemical activity in the T cell, which is apparently inhibitory and dominant over the agonist-delivered signals. These antagonists should be highly effective at much lower concentrations and may therefore be the only ones having any biological relevance. This hypothesis is supported by the data of Ferrari and colleagues, demonstrating that analogs of a hepatitis B virus peptide can efficiently antagonize T cell cytolysis when presented at a lower concentration than agonist peptide (24).

APL and Signal Transduction

While the phenomenon of partial T cell activation by APL is well documented, little is known regarding the mechanisms by which this occurs. In order to study the earliest signal transduction events stimulated upon TCR ligation, we compared the tyrosine phosphorylation patterns after TCR engagement of the Hb(64–76)/I-E^k or partially activating APL in our Th1 cells (23). The results clearly indicated that the partially activating APL, which could not activate phospholipid hydrolysis, were able to stimulate specific tyrosine phosphorylation events in the T cells. Moreover, the pattern induced by the APL was unique as compared to that stimulated by agonist or a nonstimulatory control peptide. A strikingly different tyrosine phosphorylation pattern was identified in species of 18 kDa and 20–21 kDa. Specifically, while Hb(64–76) stimulated strong bands at both molecular masses, the APL stimulated a strong band at 18 kDa and a weak but significant band at 20–21 kDa. Moreover, the nonstimulatory analog did not induce any increase in the intensity of the band at 18 kDa

above basal levels, nor any phosphoprotein at 20–21 kDa. The identification of these species as phosphorylated forms of the TCR ζ chain (pp18 and pp21) indicated that an APL was able specifically to activate membrane proximal signal transduction events leading to phosphorylation of the TCR ζ chain.

While the pattern of phospho-ζ species appearing after stimulation with the APL was different from that stimulated by agonist ligand, it was important to distinguish whether this was simply a reflection of a lower level of stimulation, or whether this APL was in fact inducing a unique signal in the T cells. A direct comparison of the phospho-ζ species appearing after stimulation with the APL or various doses of Hb(64–76) was made to address this issue. Since there was no concentration of agonist at which the pattern of ζ chain tyrosine phosphorylation resembled that seen after stimulation with the APL, we concluded that a unique signal was being delivered to the T cells by this ligand. Furthermore, this altered phospho-ζ pattern correlated precisely with the partially activating phenotype of the APL, demonstrating that APL stimulated a qualitatively different signal to the T cells than did agonist ligand. Madrenas and colleagues subsequently corroborated these results in another antigen system, demonstrating that partially activating and antagonist APL delivered an altered phospho-ζ pattern (25). They also showed that the APL could not stimulate tyrosine phosphorylation of CD3ϵ, an observation confirmed in our system (J Sloan-Lancaster, PM Allen, manuscript in preparation). Thus, partially activating APL can selectively induce tyrosine phosphorylation of the CD3 components, leading to different functional phenotypes in the T cells.

MODEL FOR APL-INDUCED PARTIAL ACTIVATION

Overview

Using the information available regarding the biochemical components and enzymes involved in T cell activation, we propose the following simplistic overview, as illustrated in Figure 2, in an attempt to explain partial activation. The TCR $\alpha\beta$ heterodimer provides the specific antigen recognition unit of T cells, while the multichain invariant CD3 complex is responsible for transducing activation signals (26–28). Since the CD3 chains have no inherent enzymatic capability, signaling is accomplished by the binding of nonreceptor tyrosine kinases and other potential effector molecules to the chains via a consensus binding module (27, 29). This immunoreceptor tyrosine-based activation motif (ITAM), with the consensus sequence $\mathrm{Yxx(L/I)x_{(6-8)}Yxx(L/I)}$, is expressed as a single copy in the cytoplasmic domains of the CD3 γ, δ, and ϵ chains, and in triplicate in the CD3 ζ polypeptide (30). Upon TCR

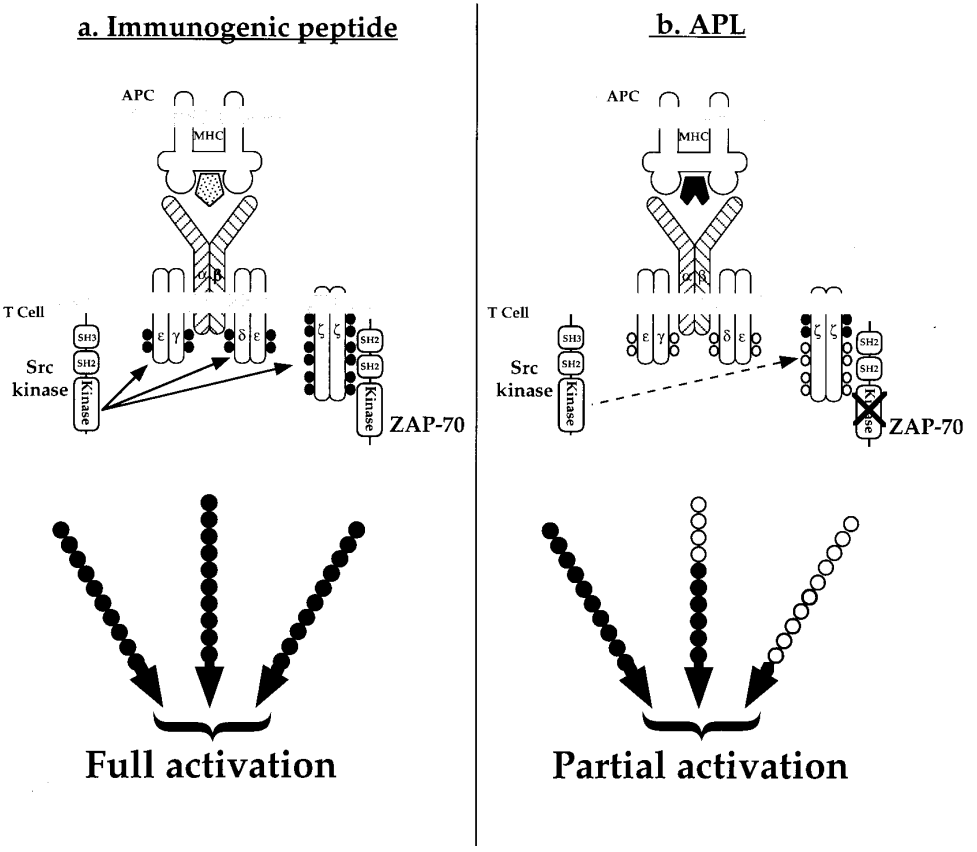

Figure 2 Model for APL-induced partial activation. (*a*) TCR ligation by immunogenic ligand leads to efficient recruitment and/or activation of the src kinases, saturated phosphorylation of all CD3 ITAMs (*filled circles*), and full T cell activation. (*b*) APL engagement causes inefficient recruitment and/or activation of src kinases, and incomplete phosphorylation of CD3 ITAMs, resulting in a lack of binding templates for some SH2-domain containing proteins. Thus, some signaling pathways remain inactive (*arrow with open circles*), others are stimulated weakly (*arrow with open and filled circles*), while others are activated normally (*arrow with filled circles*), directly dependent on the tyrosine phosphorylation state of the ITAMs.

ligation, the src kinases $p56^{lck}$ and $p59^{fyn}$ are recruited to and/or activated at the T cell contact cap (31–33). These activated kinases appear to be responsible for the subsequent phosphorylation of the CD3 ITAMs on their tyrosine residues, which then serve as binding templates for proteins containing the SH2-domain that are involved in downstream signaling events (29, 34, 35).

TCR engagement by agonist ligand should lead to efficient recruitment and activation of both src kinases, resulting in saturated phosphorylation of all CD3 ITAMs, and the subsequent binding of the full complement of downstream signaling molecules (Figure 2*a*). In contrast, we suggest that TCR ligation by an APL causes lack of recruitment and/or activation of src kinases. This results in the altered CD3 chain phosphorylation pattern witnessed upon TCR ligation by APL (23, 25). Any ITAMs remaining in the unphosphorylated state will not be able to bind SH2-domain containing proteins, resulting in a lack of recruitment of these proteins to the cross-linked TCR complex. Downstream signaling pathways dependent on those particular proteins will therefore remain inactive in the T cells (Figure 2*b*). Thus, selective signaling events will be triggered as a direct result of the phosphorylation state of the CD3 chains, which is in turn dictated by the nature of the ligand bound.

Role of the Src Kinases

If TCR tyrosine phosphorylation occurs in a hierarchial manner, both $p56^{lck}$ and $p59^{fyn}$ may be required in sufficient quantities for phosphate saturation of all available tyrosines. Alternatively, they may be responsible for phosphorylating different tyrosines in the ζ intracytoplasmic domain. Previous reports have correlated $p59^{fyn}$ association with ζ and the formation of pp21 phospho-ζ in Cos-1 cells, while coexpression of $p56^{lck}$ with ζ resulted primarily in pp18 phospho-ζ generation (36). Using this information, we propose that pp18 represents unsaturated phospho-ζ, while pp21 represents saturated phospho-ζ with all available tyrosines phosphorylated. Perhaps one src kinase primarily generates pp18, and a second is mainly responsible for converting pp18 to pp21. If binding of an APL prevents efficient recruitment and/or activation of one or the other kinase, this would result in the presence of fewer saturated phospho-ζ molecules in the T cell contact cap. Assuming that a critical number of fully active or saturated phospho-ζ molecules must be in close proximity to achieve a threshold for activation, pp18 accumulation would be inhibitory by effectively diluting out the number of pp21 molecules at the site of ligation in individual TCR contact caps. It therefore appears that the ratio of pp21:pp18 generation is important for successful T cell activation. As a result, at doses of the two ligands that induce similar levels of pp21 in the TCR population, agonist peptide would fully activate the T cells while APL would stimulate only partial signaling, due to more pp18 accumulation in the latter case. Comparison of

the levels of src kinase activity in T cells after stimulation with agonist or with partially activating APL has provided evidence to support this model. While APLs were able to activate $p59^{fyn}$ in a specific and time-dependent manner, the level of kinase activity was dramatically lower than that stimulated by a similar dose of agonist ligand. However, the contribution of $p56^{lck}$ to the activation of the T cells by the different ligands could not be addressed, because this kinase appeared to be constitutively active in the T cells (J Sloan-Lancaster, PM Allen, manuscript in preparation).

Involvement of CD3 Chains

There are six potential tyrosine phosphorylation sites in the ζ tail, making up a total of three ITAMs (37). While the exact sites of phosphorylation in the pp18 and pp21 species are not known, reports have implicated involvement of three of the four most carboxy terminal tyrosines in the generation of pp21, but not pp18 (38, 39). We therefore postulate that pp18 represents either a single species with only ITAM 1 (the most membrane-proximal) successfully phosphorylated on both tyrosines, or several species with different combinations of four tyrosines phosphorylated, producing one or two doubly phosphorylated ITAMs. In contrast, pp21 represents saturated phospho-ζ, with all three ITAMs doubly phosphorylated. The net effect should be that fewer phosphotyrosines are available in pp18 than in pp21. Thus, SH2-domain containing proteins that require several binding sites in tandem, or a particular ITAM site exclusively available in pp21, should not be recruited to the plasma membrane as efficiently after stimulation by the APL as compared to the agonist ligand. Similarly, any signaling proteins which bind exclusively to the CD3 ϵ ITAM should only be brought proximal to the membrane after engagement of agonist ligand, since APL do not stimulate CD3 ϵ phosphorylation (23, 25).

Translation to Downstream Signaling Pathways

If the phosphotyrosine state of the CD3 ITAMs dictates downstream signaling activity, then phosphorylation of fewer than the full complement of these sites upon TCR cross-linking presents several testable predictions. First, some downstream signaling pathways should remain inactive due to the absence of a binding template for the particular SH2-domain containing protein required for their initiation. We therefore investigated the tyrosine kinase activity associated with ζ after stimulation of our T cells with APL as compared to stimulatory ligand. While engagement of Hb(64–76) resulted in activation of ZAP-70 tyrosine kinase in these T cells, ligation by APL did not lead to detectable ZAP-70 kinase activity (23). Consistent with this, Madrenas et al reported that only unphosphorylated, and therefore enzymatically inactive, ZAP-70 was associated with ζ upon TCR ligation by APL (25).

Another prediction from the proposed model is that some signaling pathways should simply be lowered in activity when a T cell presents fewer phosphorylated ITAMs, perhaps due to inefficient amplification of the initiating enzyme proximal to the cell membrane. In support of this, we have investigated the intracellular Ca^{++} mobilization in our T cell clones after stimulation with the various ligands. A quantitative difference was noted, such that APL induced lower total intracellular Ca^{++} than did agonist ligand, a pattern that correlated perfectly with the partial activating phenotype of the APL. Interestingly the APL, unlike the agonist ligand, were unable to sustain the initially stimulated high intracellular Ca^{++} concentrations over the time evaluated. Instead, levels quickly dropped to a reduced but still significant concentration that was subsequently sustained, indicating a qualitative difference in addition to the quantitative difference described above (J Sloan-Lancaster, PM Allen, manuscript in preparation). These data indicate that the Ca^{++} mobilization induced by the APL is necessary and sufficient to stimulate partial activation of the T cells, but not sufficient to reach a sustained threshold level required for full activation. Finally, one might expect that some signaling pathways are activated similarly by both agonist ligand and APL, due to the presence of the initiating phospho-ITAMs for such pathways under each condition. Some of our recent investigations provide evidence to support this also, since we find equivalent activities of the MAP kinases after TCR ligation by Hb(64–76) or the Ser70 APL (J Sloan-Lancaster, PM Allen, manuscript in preparation).

SH2-DOMAIN CONTAINING PROTEIN BINDING

If one imagines the intracellular environment of a T cell as a sea of SH2-domain containing signaling proteins, as illustrated in Figure 3, then upon TCR cross-linking and CD3 ITAM phosphorylation, many binding choices become available. The factors determining which selections are made could include both 1. the actual sequences of the interacting motifs (SH2 domain and phosphorylated ITAM) and 2. the number of phosphorylated ITAMs in the environment. In the first case, signaling proteins with the highest binding affinities for individual ITAMs should successfully outcompete other signaling molecules with lower binding affinities. In a situation where few phosphorylated ITAMs of one particular variety are available, only the highest affinity signaling proteins would bind. Alternatively, if ITAM phosphorylation is maximal, some lower affinity proteins containing the SH2-domain may also be able to bind to these sites. This is referred to as the selective binding model (I). In the second case, the stable binding of signaling proteins to ITAM sequences, and perhaps their subsequent activation, may require that multiple molecules be in close proximity to one another. Such proteins would preferentially bind phospho-ζ as opposed

to the other CD3 chains, since it provides three ITAMs in sequence and therefore provides the necessary amplification property. Again, in a situation where some ITAMs of ζ remain unphosphorylated, such signaling proteins would not successfully bind and/or be activated. This is referred to as the amplification model (II). In either of these two models, which are not necessarily mutually exclusive, selective phosphorylation of individual ITAMs in the CD3 chains should allow multiple distinct combinations of downstream signaling proteins to be recruited to and/or activated at the T cell plasma membrane, with the result that the net phenotype of qualitatively different signals are transduced in the cells.

Evidence Supporting a Selective Binding Model

Observations in several receptor systems support a model of selective binding of proteins containing SH2-domains to phosphotyrosine residues. The best characterized of these is that of the platelet-derived growth factor receptor (PDGF-R), the intracytoplasmic tail of which contains numerous tyrosine residues that are sites of autophosphorylation upon ligand binding. Sequential mutagenesis studies have clearly shown substrate specificity, with unique binding sites

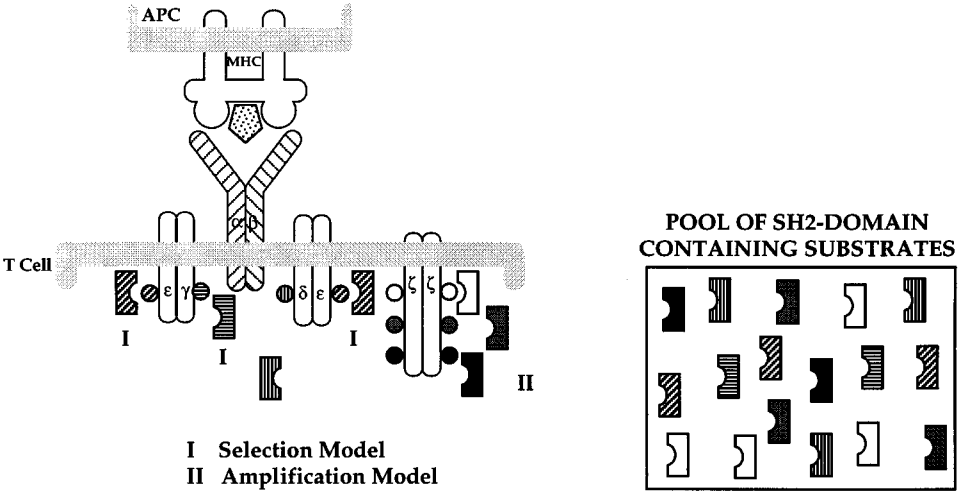

Figure 3 Model for CD3 chains binding distinct substrates. CD3 chains can bind distinct SH2-domain containing proteins by two mechanisms. The selection model (I) depends on the actual sequences of the interacting motifs and suggests that SH2-domain containing proteins bind individual ITAMs with different affinities. The amplification model (II) proposes that proteins containing SH2-domains require several ITAMs in sequence for stable binding or activation to ensue.

identified for the enzymes phosphatidylinositol-3 kinase (PI-3 kinase), GTPase-activating protein of ras (GAP), phospholipase C γ (PLC γ), and the protein tyrosine phosphatase Syp via their SH2 domains (40–42). Moreover, the Ig-α and Ig-β components of the B cell receptor complex, which are homologous to the CD3 chains, were shown to bind to distinct signaling proteins via their ITAM motifs. While both proteins could efficiently bind PI-3 kinase, only the Ig-α chain could bind the Src family kinases Lyn and Fyn (43). Recruitment of different amounts of individual enzymes to the plasma membrane in either of these receptor systems, by control of phosphorylation on different tyrosines, could potentially lead to selective activation of independent signaling pathways.

Similar observations have been made in the TCR/CD3 complex, consistent with a selective substrate binding model here also. An evaluation of the association of PI-3 kinase to CD3 ITAMs demonstrated a preferred binding pattern. This protein could only bind doubly phosphorylated ITAMs of CD3 ζ and not the other CD3 chains, and its efficiency of association was ranked ITAM ζ 1 $\gg$ ITAM ζ 3 $>$ ITAM ζ 2 (44). Moreover, our own recent studies have shown that a fusion protein containing the SH2 domain of the Shc oncogene product fused to glutathione S-transferase (GST) can specifically immunoprecipitate the pp21 but not the pp18 form of phospho-ζ from activated T cells (J Sloan-Lancaster, PM Allen, unpublished observations). Although the exact phosphorylated ITAMs represented in each of these two species is currently unknown, the data support a model in which different ITAMs of the TCR/CD3 complex recruit distinct signal transduction molecules.

A recent study investigated the relative binding affinities of the SH2 domains of ZAP-70 for individual ITAMs of CD3 ζ or ϵ, and the data generated support the selective binding model. Using immobilized GST fusion proteins containing the SH2 domains of ZAP-70, and soluble synthetic peptides with the sequences of individual CD3 ζ and ϵ motifs, the investigators demonstrated a preferential binding pattern (45). While each doubly phosphorylated ITAM peptide could bind to the tandem SH2 domains of ZAP-70, direct competition between the peptides revealed a hierarchy in the order of ITAM ζ-1 $\geq$ ITAM ζ-2 $>$ ITAM ϵ $\geq$ ITAM ζ-3. Affinity differences over a 30-fold range indicated a significant specificity of interaction of ZAP-70 SH2 domains for different phospho-ITAMs, suggesting that individual CD3 ITAM sequences likely bind distinct substrates in vivo. In contrast, results from another similarly designed study revealed no significant differences in the binding abilities of individual ITAMs for the tandem SH2 domains of ZAP-70. Instead, all individual ITAM sequences from the CD3 complex displayed similar binding abilities, except for ITAM ζ-3 which bound approximately 2.5-fold less well (46). Although the

reason for the different results from these two studies is not known, the different protocols used for affinity measurements may be an explanation. However, even if no significant selective binding of CD3 chains to ZAP-70 exists, other proteins clearly show preferential binding to individual ITAM sequences, likely a reflection of their specific SH2-domain sequences.

Evidence Supporting an Amplification Model

In support of the amplification model, several correlations between biological response and activated ITAM content have been observed. For example, a chimeric receptor containing three copies of the ζ 1 motif induced approximately threefold more NFAT-regulated activity when compared to a chimeric receptor containing only a single ζ 1 motif (47). In addition, a Tac-ϵ chimera containing only one ITAM required 10- to 100-fold greater antibody concentration to elicit IL-2 secretion than did the Tac-ζ chimera, which had three ITAMs (48). Finally, a comparison of the independent roles of CD3 ϵ and CD3 ζ in mediating various thymocyte and T cell signals provided evidence supporting an amplification model. While Tac-ϵ and Tac-ζ chimeras displayed no differences from each other or from the entire CD3 complex in mediating signals during T cell development, the Tac-ϵ chimera was less efficient at stimulating mature T cell proliferation than was Tac-ζ, and neither construct stimulated the T cells as well as the entire CD3 complex (49). Together, these results suggest that an amplification role of the CD3 ζ chain is significant for successful T cell signaling.

Existence of Both Models?

While evidence exists supporting either a selective binding model or an amplification model, it makes biological sense that both mechanisms occur in conjunction for stringent regulation of T cell activation. Indeed, results from some of our own studies are compatible with the possibility that either model may be correct, and perhaps both occur simultaneously. The demonstration that ZAP-70 kinase activity could only be detected upon TCR cross-linking by agonist ligand suggested that this enzyme binds more efficiently to pp21 than to pp18 (23). This conclusion was subsequently revised when unphosphorylated ZAP-70 was found bound to the CD3 complex after stimulation with APL as well as agonist ligand (25). Instead, the template available in pp18 appears to be insufficient to bind enough ZAP-70 molecules in close proximity to each other to initiate enzymatic activity. While these observations could be explained by a binding preference to particular ITAMs only available in pp21, they may also reflect an amplification of ZAP-70 activation when more molecules are brought into close proximity with each other.

EVIDENCE FOR DISTINCT ROLES OF INDIVIDUAL CD3 ITAMS

If different phosphorylation states of individual ITAMs in the CD3 chains dictate which downstream signaling pathways are activated, what potential roles could these different states play in T cell biology? Several independent studies support the hypothesis that individual ITAMs have specific functions during thymocyte maturation. The generation of mice lacking CD3 ζ chains highlighted the necessity of this molecule in the successful development of thymocytes, since most cells in these mice were arrested early at the double negative stage of maturation (50, 51). However, since all components of the TCR/CD3 complex are required for surface expression, these studies could not distinguish whether the lack of development was due solely to a lack of TCR on the cell surface, or whether CD3 ζ also played a critical role in the signaling processes during development. TCR expression was subsequently restored in these ζ-deficient mice by reconstituting with transgenic ζ chains that partially or completely lacked sequences required for signal transduction (52). The ζ chain was shown to play a role in thymic development by promoting TCR surface expression, but ζ-mediated signals were not essential since TCRs that contained signaling-deficient ζ chains promoted T cell maturation also. However, the efficiency with which the transgenic ζ-chain variants reconstituted thymocyte development paralleled the number of ITAMs that were present in the protein, implying that ζ-mediated signals are important and perhaps function in signal amplification. Consistent with this, mice lacking CD3 ζ, but not its alternatively spliced partner η, displayed a phenotype suggesting an amplification role of the ITAMs in T cell signaling (53). Here, mice lacking only expression of ITAM ζ 3 displayed greatly reduced numbers of both thymocytes and peripheral T cells, with fivefold-lower surface levels of TCR than those of normal mice. Interestingly, the functional responsiveness of the peripheral T cells was also impaired, as evidenced by the reduced proliferative response upon TCR engagement. These data also supported a model of selective roles of individual ζ ITAMs in TCR signaling, both developmentally and in the mature T cell population, whether it be via the selective binding or the amplification model.

The phenotypes created by overexpression of CD3 ζ or CD3 ϵ in transgenic mice also supported the proposal of independent functions of ITAMs during thymic development. In one case, mice were generated that overexpressed either full-length ζ (FL ζ), η, or a truncated ζ chain containing only ITAM ζ 1; and the functional consequences of high surface levels of these proteins compared (54). Unexpectedly, overexpression of FL ζ, but not η or ITAM ζ 1, caused

premature termination of RAG-1 and RAG-2 expression, prevented productive rearrangement of the TCR α and β genes, and blocked entry of thymocytes into the CD4/CD8 developmental pathway (54). Similarly, an analysis of the role of CD3 ϵ expression during thymocyte development suggested that overexpression of this CD3 chain also arrested T lymphocyte development early during ontogeny (55). A correlation between the copy number of transgene expression and stage of maturational block was noted, such that increasing levels of CD3 ϵ on the thymocyte surface blocked the cells at an earlier stage of development. The importance of the ITAM motif was evident because mice containing a CD3 ϵ transgene lacking most of the intracytoplasmic domain contained normal numbers of T cells. Together, these data support a model of independent roles of CD3 ITAMs during thymocyte development and are compatible with the individual ITAMs binding either individual SH2-domain containing substrates or different quantities of shared substrates as a mechanism for their specific functions.

In addition to the above studies, a recent report has implicated specific requirements for different components of the CD3 complex at distinct levels of maturity in the peripheral T cell population for successful T cell activation (56). Transgenic mice were constructed bearing a chimeric molecule composed of the intracytoplasmic domain of CD3 ζ, transmembrane portion of CD8 α, and extracellular domain of a single chain Ab (Fv) portion specific for human CD3 ϵ. This produced a system in which the signaling efficiency of a TCR containing the entire CD3 complex could be directly compared to one containing only a single CD3 ζ molecule (56). In vitro stimulation of naive T cells via the chimeric molecule, unlike cross-linking of the endogenous CD3 complex, failed to stimulate T cell proliferation, cytotoxic function, or intracellular Ca^{++} fluxes. Intriguingly, T cells from these mice previously activated in vivo, or in vitro via the intact CD3 complex, were subsequently able to be activated through the chimeric CD3 ζ molecule. These observations strongly support the hypothesis that the signaling requirements of peripheral T cells are dictated by the activation state of the cells prior to ligand binding, and they further demonstrate the significant influence individual CD3 components can have in the success of T lymphocyte activation.

TRANSLATION OF STRUCTURAL CHANGES IN LIGAND ACROSS THE PLASMA MEMBRANE

While all the above proposals may help in understanding how partial TCR signaling can occur, one crucial question remains unaddressed. That is, how are subtle changes in the exogenous binding to the $\alpha\beta$ recognition unit being

transmitted across the membrane to result in the altered intracellular signaling events witnessed? Since T cell recognition involves the binding of several distinct TCR contact residues of the peptide ligand, two non–mutually exclusive possibilities exist for how creation of a subtle change in a TCR contact site leads to partial activation. These are illustrated in Figure 4 as either (*a*) a failure to assemble all the necessary molecules in the T cell contact cap (Oligomerization Model), or (*b*) a failure to induce a required conformational change in the TCR (Conformational Model). Changing the ligand subtly at a TCR contact site may change the composition of the contact cap between the T cell and APC. This changed composition could include fewer TCR/CD3 complexes, less CD4, CD28, LFA-1, or various other molecules, with the subsequent signals integrated differently, perhaps by abrogating the recruitment and/or activation of one signaling molecule and not the other. The successful oligomerization of all necessary components in a functional contact cap may then be a prerequisite for induction of a conformational change in the TCR complex. In support of this, Janeway's group demonstrated using anti-TCR antibodies that both receptor cross-linking and a change in conformation must occur for successful activation to ensue (57). Similarly, by modifying the structural interaction of the ligand at a TCR contact site, insufficient receptor cross-linking and failure to induce a conformational change may occur, leading to partial activation. While it seems feasible that APL have a lower affinity for the TCR than native ligand, an increase in affinity cannot be ruled out. One investigation demonstrated no affinity difference between agonist and variant peptide, but instead a higher dissociation rate of the APL (58). Thus, affinity differences or kinetic changes may lead to altered stoichiometry or a lack of TCR conformational change, resulting in partial activation.

A recent study described an assay system in which the stoichiometry of peptide-MHC/TCR interaction in specific conjugates could be precisely measured by assessing the level of TCR downregulation upon stimulation with known numbers of ligand molecules (59). By titrating the dose of agonist ligand used to pulse the APC, the investigators were able to show that a large percentage of TCR were specifically engaged by relatively few peptide-MHC complexes, indicating that each peptide-MHC complex must engage a large number of TCR in successive rounds. Furthermore, the production of IFN-γ by the T cells correlated with the number of TCR engaged. When partial agonist APL were tested in the assay system, a similar relationship between TCR engagement and biological response was found. These results indicated that APL may behave as partial agonists because they trigger fewer TCR than native ligand and may prevent aquisition of the full complement of receptors at the contact cap or lack of a TCR conformational change from occurring.

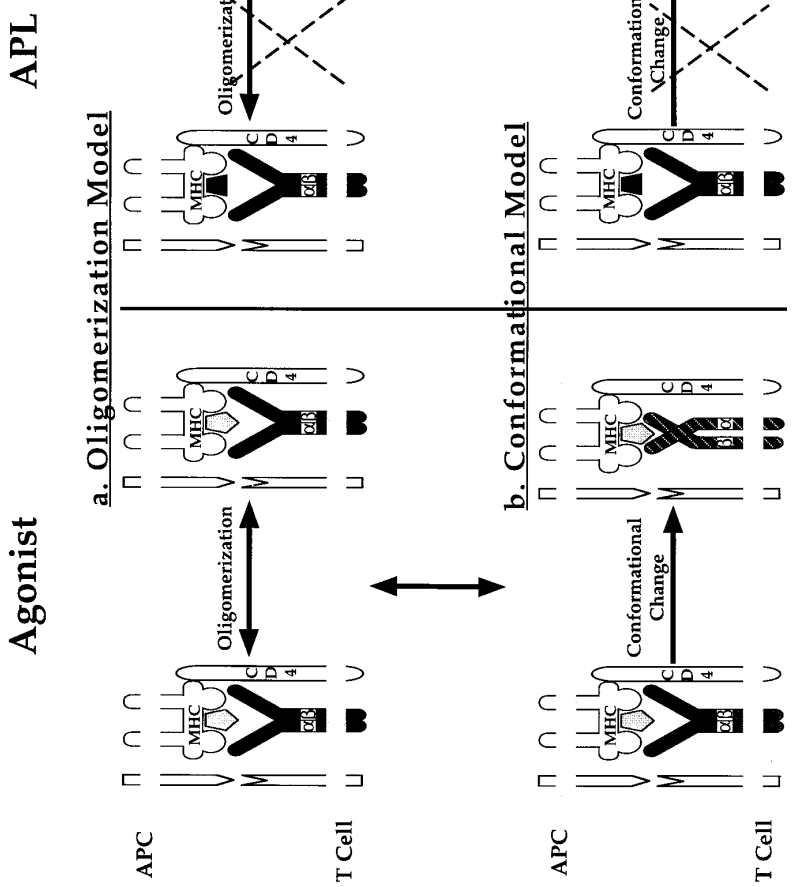

Figure 4 Translation of ligand engagement across the T cell plasma membrane. TCR engagement by agonist ligand may allow assembly of a critical number of TCR molecules and other coreceptor molecules at the T cell contact cap (*a*, oligomerization model) and/or induce a conformational change in the TCR (*b*, conformational model), resulting in the successful activation of the cells. APL may fail to assemble all necessary components of the T cell contact cap (*a*), or fail to induce the conformational change (*b*), leading to only partial activation of the T cells. The vertical arrow indicates that a conformational change may be required before or after TCR oligomerization.

BIOLOGICAL RELEVANCE OF ALTERED PEPTIDE LIGANDS

Many models of in vivo T cell activation are compatible with a selective signaling model, suggesting that naturally occurring APL for any TCR exist. Such natural APL could be constructed from one's self-antigens and the resulting repertoire of endogenous peptides, or they could be designed by pathogens to escape immune attack from the host. Recently, observations from many studies have implicated several roles for such analog peptides in various aspects of immune responses. Collectively, these data suggest the existence of naturally occurring APL from the endogenous peptide repertoire, which actively participate in the development and shaping of T cell immunity. Supporting this theory, two recent studies have clearly shown the relevance of endogenous APL for individual TCRs, and the intriguing data from these reports are discussed later in the review.

APL Role in Thymic Development

Thymic education results in the maturation of T lymphocytes recognizing foreign antigens, but not self-proteins, bound to self-MHC molecules, and it involves two independent selection processes. Positive selection allows cell survival and differentiation, while negative selection leads to cell death by apoptosis (60, 61). Both processes require the successful engagement of the TCR with a peptide-bound MHC molecule (62), indicating that differences in the peptide's interaction with thymocytes must dictate the fate of the cell. The recent design of in vitro culture models allowed a direct assessment of the possible involvement of APL in thymic development, and the data indicate a potential role of such ligands, supporting an affinity/avidity model of thymic maturation.

By culturing fetal thymic lobes from class-I MHC-deficient, TCR transgenic mice with mixtures of peptides, two groups analyzed the involvement of peptide in $CD8^+$ T cell thymic development in vitro (63, 64). These studies elegantly demonstrated that peptide variants, which formed a subset of specific antagonists for mature T cells expressing the same TCR, could successfully positively select thymocytes when the variants were included in the fetal thymic organ cultures. Subsequent analyses of the role of agonist or partial agonist ligands in thymic development revealed that, while low concentrations of these peptides supported positive selection, higher concentrations resulted in thymic deletion (63–65). This suggested that the outcome of the interaction between a T cell and its ligand depends both on the affinity of the TCR/MHC-peptide complex and on the number of TCRs engaged (66).

In parallel, other groups have assessed the role of specific peptides in the development of $CD4^+$ T cells. For example, APL that could only partially activate

a mature $CD4^+$ T cell clone could nevertheless efficiently delete thymocytes expressing the same TCR, indicating that perhaps thymocytes have a lower threshold of activation requirement than do mature T cells (67). Furthermore, endogenous expression of a TCR antagonist peptide in a TCR β transgenic mouse resulted in the deletion of T cells with high avidity for agonist ligand, while allowing survival of T cells with lower avidity for the same ligand (68).

The above data emphasize that different signals are involved in positive versus negative selection, and they provide a possible mechanism whereby this can occur. One can imagine that peptide interaction with thymocytes at different stages of maturation could stimulate different signaling pathways. This could result from the overall avidity of the interaction between the T cell and APC, and would be the summation of TCR affinity for ligand, concentration of ligand presented, and the participation of other adhesion molecules on the cell surfaces. Since thymocytes change both surface levels of TCR and other molecules as they progress through thymic development, interaction with the same peptide at different stages could result in different functional consequences. This should be due to the signal transduction processes initiated, which in turn would be dictated by the initial tryosine phosphorylation state of the various CD3 ITAMs.

What then is the relationship between mature T cells and their positively selecting ligand in the periphery? Perhaps such an interaction is analogous to that between APL and T cells, such that these ligands cannot stimulate full activation of peripheral T cells. In one study, thymocytes educated by a particular peptide were shown to be specifically unresponsive to it by decreasing surface CD8 levels (69). Furthermore, transgenic $\alpha\beta$ T cells specific for gp(33–41)/H-$2D^b$ from lymphocytic choriomeningitis virus (LCMV) were specifically unresponsive to the self-ligand Mls-1a while still retaining the ability to be activated by agonist ligand (70). This suggests that low avidity interactions with self-ligands may induce unresponsiveness to self-molecules by increasing the resting threshold of the T cell, and that foreign antigenic peptides with higher affinity for such TCR are able to stimulate this threshold of activation level in the mature T cell population.

Implications for APL in the Periphery

SHAPING OF IMMUNE RESPONSES TO PARTICULAR ANTIGENS The predisposition of certain individuals to autoimmune diseases or allergies indicates that biological factors shared by them contribute to their susceptibility. Indeed, strong correlations between expression of particular HLA alleles and disease susceptibility are well documented. However, multiple other factors are clearly involved and likely include both genetic and environmental influences. We propose that one's repertoire of endogenous peptides not only influences susceptibility to disease, but also plays a significant role in the shaping of immune responses

to all foreign challenges, as well as dictating vaccine efficiency within individuals. This could be accomplished either by an involvement of self-peptides during thymocyte development as discussed above, or by active participation in the regulation of mounting immune responses in the periphery. Endogenous peptides recognized by mature T cells as APL should be able to function as such in vivo, and thus they should influence responses activated against foreign antigens, e.g. by antagonism or anergy induction or by influencing the phenotype of the T cell population being activated. What is the existing evidence in support of this proposal?

Modulation of cytokine pattern by APL One elegant study recently demonstrated that antagonistic APL were able to partially activate Th0 clones in a way that caused dramatic changes in their functional phenotype (71). While presentation of agonist ligand to myelin basic protein (MBP)–specific Th0 clones stimulated production of IL-2, IL-4, IL-10, and IFN-γ, none of these cytokines could be detected after TCR ligation by an antagonistic APL. Intriguingly, engagement of the APL alone induced production of TGF-β from the T cells, a cytokine that was not produced upon agonist stimulation (71). These data clearly showed that APL engagement can activate different signals than agonist ligand, which can result in a dramatic change in the functional phenotype of the T cells. The biological significance of this as a possible way of regulating specific T cell immune responses is highlighted by the observations that TGF-β administration to mice can ameliorate EAE disease severity, and that suppression of EAE is associated with the generation of T cells that secrete TGF-β (72–74).

Viral antagonism While viruses have long been known to utilize several strategies to escape attack by host defense systems, two reports documented a novel means of immune evasion, that of viral antagonism (24, 75). Naturally occurring variants of antigenic epitopes were identified, which may act as antagonists in vivo, since the corresponding peptides specifically prevented a CTL response directed against the viruses in vitro. Moreover, these peptide variants were able to function as antagonists when expressed as proteins and thus when synthesized in the target cell. These results strongly suggest that naturally occurring APL can perform as efficient antagonists, and thus they could significantly influence specific immune responses.

Ability of endogenous APL to antagonize a T cell response To formally test the above hypothesis, we have recently studied the ability of endogenously synthesized peptide-MHC complexes to function as TCR antagonists (K Vidal, BL Hsu, C Williams, PM Allen, manuscript in preparation). Generation of TCR-β chain transgenic mice created a system in which an APL now stimulated

primary reactivity of the resulting T cells when paired with endogenous α chains, while the original agonist peptide Hbd(64–76) functioned as an antagonist of the response (68). This allowed us to examine whether endogenous Hb^d/I-E^k complexes can antagonize peripheral T helper cell responses, since the immunogenic Hbd epitope is processed and presented by many APC populations in mouse strains expressing the appropriate MHC molecules and the Hb^d allele (76). The initial data revealed that endogenous Hb^d/I-E^k complexes were unable to antagonize cytokine production of a T cell hybridoma derived from the transgenic mice. However, by increasing surface levels of endogenously synthesized complexes using several strategies, efficient antagonism of the induced cytokine response was achieved. These data indicated that the normal levels of presentation of endogenous peptide complexes limit their ability to function as antagonists. In a pathological situation in vivo, where many inflammatory mediators and cytokines enhance antigen presentation, such endogenous APL may be capable of downmodulating specific T cells against agonist ligands.

Maintenance of T cell memory The role of specific antigen in the maintenance of T cell memory has remained a controversial issue in the literature (77). Two independent reports presented compelling evidence that memory $CD8^+$ T cells survive, both phenotypically and functionally, in the absence of specific antigen (78, 79). However, since these T cells express increased surface levels of some adhesion molecules and IL-2R compared to their naive counterparts, they may require a lower threshold of activation to achieve full stimulation. Perhaps, therefore, some cross-reactive ligands, which have a lower affinity interaction than the antigenic peptide, could stimulate memory T cells but not naive T cells expressing the same TCR. Thus, naturally occurring APL may play a significant role in the longevity of specific immune responses.

EVIDENCE FOR EXISTENCE OF ENDOGENOUS APL

We recently assessed the ability of a TCR to recognize a variety of different ligands, to define the rules for such interactions, and to establish how much sequence dissimilarity can exist between agonist ligand and partially activating APL (80). Surprisingly, the findings revealed that a peptide with as few as the single primary TCR contact amino acid in common with the agonist ligand, in combination with tolerable substitutions at the other TCR and MHC contact sites, was sufficient to partially activate the T cells. To assess if natural agonists or partial agonists exist in the endogenous peptide repertoire, a search of the databases was made that identified both a natural agonist and a partial agonist peptide for these T cells. This report thus demonstrated that self-peptides have the potential to partially stimulate T cells expressing appropriate TCR, and

they may play a significant role in various aspects of T cell biology. Moreover, the nonhomologous nature of many partially activating peptides highlights the flexibility of the specificity of TCR recognition.

Since activation of autoreactive T cells is a critical event in the induction of autoimmunity, the question arises as to how these T cells are initially activated. Much evidence indicates bacterial or viral infection is a prerequisite of many autoimmune diseases; this implicates cross-reactive peptides from pathogens in the primary stimulation of self-antigen–specific T cells. Using such information, Strominger and colleagues tested the ability of bacterial and viral peptides to stimulate a panel of T cell clones derived from patients suffering from multiple sclerosis (MS) and recognizing MBP(84-102)/HLA-DR2^{b} (81). Basing their search on peptides that possess accommodating amino acids at the established MHC and TCR contact sites, the investigators were successful in finding several bacterial or viral peptides capable of stimulating three T cell clones. Moreover, a stimulating peptide from EBV was processed naturally in an EBV-transformed B cell line and was able to activate the appropriate T cells. Surprisingly, these cross-reactive peptides did not bear a major degree of sequence homology to the agonist ligand, indicating that T cell mimicry results from TCR engagement by ligands with enough structural similarity to induce potent T cell activation, regardless of primary amino acid identity.

CONCLUSIONS

This manuscript was designed to provide a comprehensive overview of the concept of APL, their structural and functional relationship to agonist ligands, and their potential roles in various aspects of T cell biology. Many recent and exciting reports have provided compelling evidence that such ligands can exist for any individual T cell, highlighting the relevance of their activation properties to the shaping of T cell–mediated immunity. These data validate the concept of partial T cell signaling and emphasize that T cell activation is not an “all-or-none” phenomenon, but rather involves a collection of signaling modules that can be selectively triggered upon TCR engagement. We propose that these signaling modules are downstream of the TCR/CD3 complex, and that their activation is dependent on the nature of the initial tyrosine phosphorylation events on the CD3 proteins, which in turn is directly dictated by the nature of the ligand bound. This dynamic and rapidly developing field will most likely continue to identify APL as active participants in many T cell biological phenomena, and to reveal the relative importance of individual signal transduction pathways in isolation as opposed to being encompassed in the normal intracellular biochemical homeostasis created upon full T cell activation.

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EARLY T LYMPHOCYTE PROGENITORS

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ABSTRACT

The earliest steps along the pathway leading to T cells in mice and humans are reviewed. These are the steps between the multipotent hemopoietic stem cell (HSC) and the fully committed precursors undergoing T cell receptor (TCR) gene rearrangement. At this level significant differences between adult and fetal lymphopoiesis have been demonstrated. The extent of lymphoid commitment of precursors within bone marrow is still unresolved, although HSCs clearly undergo developmental changes before migration to the thymus. Both multipotent and T-restricted precursors have now been isolated from fetal blood, suggesting both may seed the thymus. Within the thymus, several minute but discrete populations of T precursors precede the stage of TCR gene rearrangement. They include precursors that are not exclusively T-lineage committed, although they are distinct from HSCs. These precursors have a potential to form NK cells, B cells, dendritic cells, and sometimes other myeloid cells. Some factors that control early lymphoid development are discussed, including IL-7 and the Ikaros transcription factors. These will eventually help to clarify the process of T-lineage commitment.

INTRODUCTION

All lymphoid cells derive, ultimately, from multipotent hemopoietic stem cells (HSCs) found in the liver during fetal life and then in the bone marrow of

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the adult. Because such stem cells can be considered as "early T lymphocyte progenitors," we must define the territory of this review. We begin calling stem cells "early lymphoid progenitors" when they have lost at least some of their capacity to form blood cells other than lymphocytes. We cease calling T lymphocyte progenitors "early" once they have completed rearrangement of the genes specifying either the β or the γ chain of a T cell antigen receptor. We therefore review data on a range of progenitor cells from those that have been called lymphoid stem cells to those termed pro-T cells. Our interest is in the process of T lymphocyte lineage commitment.

DIFFERENCES BETWEEN FETAL AND ADULT T LYMPHOPOIESIS

The lymphocytes formed in adult life are derived from different sets of HSCs than those formed in the embryo. This is most evident for T cell development in the thymus where the site of lymphoid development is well separated from the source of stem cells. The classic studies of Le Douarin and colleagues (1, 2) demonstrated that the avian thymus is colonized in succession by discrete waves of stem cells. A similar situation occurs in the fetal mouse thymus, where Jotereau et al (3) have shown that the stem cells or lymphoid precursors seeding the fetal thymus between days 10 and 13 are the source of the T cells produced by the thymus up to the first week after birth, while the stem cells seeding the thymus after day 13 of fetal life produce a second generation of T cells that begins to displace or dilute the first generation from day 7 after birth. Further seeding of the thymus occurs in adult life (4, 5).

The implications of these differences in stem cell origin for subsequent lymphopoiesis would be minimal unless the fetal and adult lymphoid developmental programs differ. Such a possibility was happily ignored by many workers who used the analysis of thymuses harvested on successive days of embryonic life to deduce the successive steps of both embryonic and adult T cell development. In the main this optimistic approach was effective; most of the key events are common to the embryo and the adult. However, recent work has highlighted some important differences. The striking finding is that some of these reflect precommitment or restrictions on developmental options at the level of an otherwise multipotent HSCs.

The clearest example is $\gamma\delta$ T cell generation in which during ontogeny successive waves of $\gamma\delta$ T cells exit the thymus, destined to seed different peripheral tissues. The successive waves are characterized by differing T cell antigen receptor (TCR) gene utilization (6). In embryonic mice the first wave of TCR-gene rearrangement in the thymus leads to Vγ3-bearing cells, which

become the Thy-1$^+$ dendritic epidermal $\gamma\delta$ cells found in the skin of the adult (7). Ikuta et al (8) have shown, by purifying HSCs from fetal liver or adult bone marrow and using these to repopulate fetal or adult thymuses, that Vγ3$^+$ T cell development requires not only a fetal thymus environment, but also fetal HSCs. Adult stem cells have apparently lost the potential to form Vγ3$^+$ T cells. The basis of this altered developmental potential has not yet been elucidated.

Another difference between embryonic and adult lymphoid development is the presence of the enzyme terminal deoxynucleotide transferase (TdT) in early T and B lineage cells of the adult but not of the embryo. This enzyme is responsible for template-independent (N region) nucleotide addition during antigen-receptor gene rearrangement; this introduces an additional level of diversity into both the TCR and immunoglobulin (Ig) repertoire (9–11). Lymphoid cells produced during adult life have this additional N region diversity, while lymphoid cells produced during embryonic life do not; however, adult mice lacking TdT because of a mutated gene have an "immature lymphocyte repertoire" with few N nucleotides (10, 11). When fetal thymus cultures were reconstituted with adult or with embryonic stem cell sources, both allowed Vγ4$^+$ T cell development, but the Vγ4-Jγ1 transcripts from the adult precursors had modified junctions with N nucleotide insertion (12). The generality of this result still needs formal testing for other TCR genes. However, it is now reasonable to propose (13) that the ability to express TdT and perform N nucleotide insertions represents another difference in developmental potential already determined at the multipotent HSC level. Many other differences between adult and embryonic lymphopoiesis may be secondary consequences of this difference in TdT expression.

One possible candidate is the propensity of adult thymic CD4$^-$8$^-$ precursor cells to generate many more CD4$^+$ than CD8$^+$ T cells, in contrast to early embryonic CD4$^-$8$^-$ precursors where the T cell progeny are produced in nearly equal levels. Adkins (14) has demonstrated, by seeding embryonic precursors into adult thymuses and vice versa, that the ratio of CD4$^+$ to CD8$^+$ progeny is determined by the precursor cells themselves rather than by the thymic environment. She has suggested these precursor cell differences reflect, in turn, differences in the successive waves of stem cells populating the thymus (14). Since both CD4 and CD8 T cells are produced only after a very stringent selection for immunological specificity, it is possible that the difference in relative production reflects the presence or absence of TdT in the precursor cells. By this argument the higher N region TCR diversity associated with the adult TdT$^+$ precursors in some way leads to more class-II major histocompatibility complex–restricted receptors appropriate for CD4$^+$ T cells.

EARLY T PROGENITORS AND STEM CELLS IN BONE MARROW

The HSCs of adult mouse and human bone marrow are heterogeneous in capacity to produce long-term reconstitution of the hematopoietic system as well as in cell cycle status, physical properties, and surface antigen expression (13, 15–18). The stem cell population appears to be separable into subpopulations representing stages of differentiation between the most primitive long-term reconstituting cells and the lineage-committed progenitors (18). A major issue is whether commitment to a given lymphoid lineage is an abrupt process or whether there exist intermediate self-renewing stem cells that are common to all lymphoid lineages, but no longer able to form myeloid or erythroid cells. Such a "common lymphoid-restricted" stem cell has been an almost mythical entity, prominent in wall-charts of hemopoiesis but elusive when its isolation from bone marrow is attempted.

The surface markers that have proven useful for the positive characterization of multipotent HSCs (Sca-1^+ Thy 1.1^{lo} c-kit$^+$ in the mouse; CD34^+ in the human) are also expressed by immature cells of various lineages. To obtain pure stem cells it is necessary to combine positive sorting for these markers with a procedure to exclude cells bearing lineage-restricted surface markers (Lin$^+$ cells). However, one might hope to isolate a lineage-restricted stem cell on the basis of early surface expression of one of these lineage markers. Although stem cell activity had been reported in the "Lin$^+$" fraction of mouse bone marrow, a careful reexamination of the finding has indicated that almost all long-term reconstituting activity is within the Sca-1^+ Thy 1.1^{lo} and Lin$^-$ population (17–19). Perhaps this is not an absolute phenotype, since some stem cell activity is detected in Mac-1^{lo} and CD4^{lo} fractions of normal mouse marrow (17, 19), and CD4^{lo} Gr-1^{lo} progenitors have been detected in 5-fluorouracil–treated marrow (20). However, these stem cells bearing low levels of the lineage markers all appear to be multipotent.

It is possible that some markers other than those recognized by the usual "anti-Lin" cocktail of antibodies could serve to delineate a lymphoid-restricted stem cell. For example, the ER-MP12 antigen is on murine bone marrow cells with thymus repopulating activity (21), but it is not yet clear whether multipotent HSCs also bear this antigen. The Joro 37-5 and Joro 75 antibodies recognize Thy 1^+ Lin$^-$ T-progenitor cells in murine bone marrow, but these appeared to be T-lineage restricted and unable to produce B cells (22). The antigen Sca-2, which is present on T precursors within the thymus (see later), but absent from long-term repopulating bone marrow HSCs, has been used by Antica et al (23) to search for thymus-colonizing cells in bone marrow. Although a new Sca-2^+ T-precursor population distinct from most HSCs was isolated, it was not

lymphoid restricted in its reconstitution potential. This result fits with evidence that the multipotent stem cells with rapid (3 wk) thymus colonizing activity after intravenous transfer are those more active cells with a high capacity to take up rhodamine 123 ($Rh123^{hi}$), and not the more quiescent long-term repopulating $Rh123^{lo}$ bone marrow HSCs (24, 25). Overall, these studies suggest that murine HSCs do undergo distinct phenotypic changes before leaving the bone marrow to seed the thymus. The extent of T commitment in bone marrow is not yet clear, but to date no population has been isolated with a capacity to make T and B lymphocytes but not myeloid or erythroid cells. Although the search for a lymphoid-committed stem cell in murine bone marrow has been unrewarding, it should be noted that even traces of primitive, multipotent HSCs in a lymphoid-committed precursor fraction would be enough to obscure evidence for lymphoid restriction.

Studies on adult human bone marrow have shown more promise in delineating a population of lymphoid-restricted precursors. Extending earlier evidence (26) that the $CD34^+$ fraction includes lymphoid progenitors, Galy et al (27) have subdivided the $CD34^+$ Lin^- population. Additional markers were employed, including Thy 1 and CD45RA, the latter chosen because early thymocytes are $CD45RA^+$. The developmental potential of the subpopulations of $CD34^+$ Lin^- cells was assessed by reconstitution of human bone and thymus fragments implanted into SCID mice as well as by various in vitro culture systems. The more primitive, multipotent HSCs were found to be $CD34^+$ Lin^- Thy 1^+ and $CD45RA^-$; these were able to repopulate both the thymus and the bone marrow fragments. However, a $CD34^+$ Lin^- Thy 1^- $CD45RA^+$ fraction, considered to be the progeny of HSCs, lacked ability to repopulate bone marrow, to maintain $CD34^+$ cells in culture, or to form erythroid cells, but the fraction had enhanced T-progenitor activity in the thymus fragment reconstitution assay. It would be of interest to know if these cells contain TdT, since a $CD34^+$ TdT^+ bone marrow population has been described by Gore et al (28) and considered to represent early lymphoid precursors, although functional assays were lacking. Recent extensions of these studies by Galy and colleagues (AHM Galy, personal communication) have led to the identification of a "lymphoid progenitor" population with a capacity to form T cells, B cells, natural killer (NK) cells, and interdigitating dendritic cells (DC), but not to form normal myeloid, erythroid, or megakaryocytic cells. This suggests development within the bone marrow of a common lymphoid progenitor, if the term can be extended to include certain DC. However, clonal studies will be required to eliminate the possibility that the population is a mix of individual unipotent progenitors (either T, or B, or NK or DC committed) with identical surface phenotype. At this stage it is not clear whether mouse and human differ in their bone marrow stem cell populations, or

whether the markers and the assays used for human bone marrow have simply proven more effective at revealing lymphoid commitment.

EARLY T CELL PROGENITORS AND THYMUS SEEDING CELLS IN BLOOD

The anatomical separation of the source of stem cells from the site of T lymphopoiesis in the thymus provides a useful inbuilt segregation of two levels of T-lymphocyte development. However, the issue of whether thymopoiesis in the adult requires a continuing input of progenitors from bone marrow, or whether it is self-sustaining, has long been a point of dispute. If HSCs are injected directly into an irradiated thymus, they produce long-term reconstitution and persistence of stem cells able to form spleen colonies (29). However, this mode of HSCs entry into the thymus is obviously artificial. Donskoy & Goldschneider (5) have made a careful reexamination of the issue of thymic seeding in the adult, employing Thy-1 alloantigen-disparate parabiotic mice. They concluded that thymopoiesis is dependent on a low but continuing input of blood-borne progenitor cells, in agreement with the earlier assessment of Scollay (4)

What form of progenitor enters the thymus from the bloodstream? Until recently attempts to answer this question were indirect, relying on the characterization of the cells with thymus seeding capacity in the bone marrow or on the characterization of early T precursors in the thymus. As discussed above, the data from analysis of bone marrow is ambiguous, although it provides some good candidates for a thymic seeding role. However, even the presence of T-restricted progenitors in bone marrow does not prove they will make the transit to the thymus. On the other hand, the analysis of the earliest T-progenitors in the thymus also provides incomplete, if important, information. Within the embryonic mouse thymus there are precursors capable of forming both B cells and macrophages as well as T cells (30, 31). Within the postnatal human thymus there are $CD34^+$ cells with myeloid as well as T cell–developmental capacity (32, 33). Within the adult mouse thymus, early T-precursor populations can form as well as T cells, B cells, NK cells, and DC (34, 35). All this suggests the thymus, or at least the embryonic thymus, is seeded by some form of multipotent stem cell (36).

In the case of the adult mouse thymus, there are two arguments against seeding with a multipotent HSCs. First, although T-lineage cells are produced when pure bone marrow HSCs are injected intrathymically, so are granulocytes, which are not normally found in significant numbers in the thymus (37). Second, very few multipotent stem cells (such as cells forming spleen colonies) can

be extracted from the adult thymus itself. However, if the actual rate of multipotent HSC seeding is very low, and if they become restricted in developmental potential soon after entry, these arguments are negated. The available evidence does suggest a very low seeding rate. Studies on parabiotic mice indicate that intrathymic precursors are being replaced at a rate of only 2–3% per day (5). In addition, the number of "environmental niches" available for seeding the adult thymus has been estimated to be only around 200, most of which are occupied in a normal, nonirradiated animal (37).

It is evident that a more direct approach is required. The appropriate place to search for the progenitor cells seeding the thymus from the bloodstream is in the blood itself, and the appropriate assay for the seeding process is intravenous rather than intrathymic transfer. Rodewald et al (38) have responded to this challenge by characterizing the T-progenitor populations in fetal blood at day 15.5 of gestation. In some compensation for the technical demands of handling minute samples of blood, most of the leukocytes present at this age are progenitor cells. Two progenitor populations with thymic reconstitution activity were isolated. One, Thy-1$^-$ c-kit$^+$, was able to colonize bone marrow as well as thymus and was a multipotent HSC because it generated T cells, B cells, myeloid cells, and erythroid cells. The other, Thy-1$^+$ c-kit^{lo}, was a T-restricted progenitor because it was unable to reconstitute bone marrow or to form B cells or other hemopoietic lineages. No common lymphoid-restricted progenitor was detected. Although these experiments still do not directly show seeding from the blood to the thymus in the embryo itself, this seems likely since these two different T progenitors at different stages of development are in the bloodstream and have a demonstrated capacity for thymus homing. These experiments also demonstrate that T-lineage commitment can occur before entry into the thymic environment. Similar experiments are now required to characterize the cells in the blood of adult mice and humans that have thymus seeding ability.

EARLY T PROGENITORS IN THE THYMUS

Adult Mouse Thymus

The search for early T precursors in the adult murine thymus began with a subdivision of the 2–5% "triple negative" (CD3$^-$4$^-$8$^-$) population. Within this population it was evident that expression of the α chain of the IL-2 receptor (CD25) by about half the cells marked an important stage of development (39, 40), although the function of this low affinity chain is still obscure. However, even the CD25$^+$3$^-$4$^-$8$^-$ subset is heterogeneous; the additional markers CD44 (Pgp-1) and c-kit (the receptor for stem cell or steel factor) are required to segregate the individual steps of development (41, 42). Godfrey, Zlotnik and

colleagues (42, 43) have recently characterized two $CD25^+$ populations on the mainstream pathway leading to $\alpha\beta$ T cells, namely $CD3^-4^-8^-25^+44^+$ c-kit^{hi} and $CD3^-4^-8^-25^+44^-$ c-kit^{lo} (populations C and D of Figure 1 respectively). The former has Cβ genes in germline state whereas the latter is extensively rearranged (42); using a polymerase chain reaction (PCR) assay, we have now shown the same applies for the Vβ2, Dβ2 and Jβ2 genes (L Wu, unpublished). Rearrangement of the TCR β chain therefore occurs quite abruptly within the $CD25^+$ subset, not gradually along with surface marker changes as we had previously deduced (40). Further development after β-chain rearrangement is marked by loss of CD25 (population E of Figure 1), then upregulation of CD4 and CD8 and rearrangement of the TCRα chain (reviewed in 43, 44). Control of exit from the $CD25^+$ stage is probably mediated by an association of the TCR-β chain with the newly discovered pTα chain and the CD3 complex (45).

Other, numerically minor, T cell lineages developing in the thymus appear to branch off at about the point of CD25 expression (43) (Figure 1). One is the

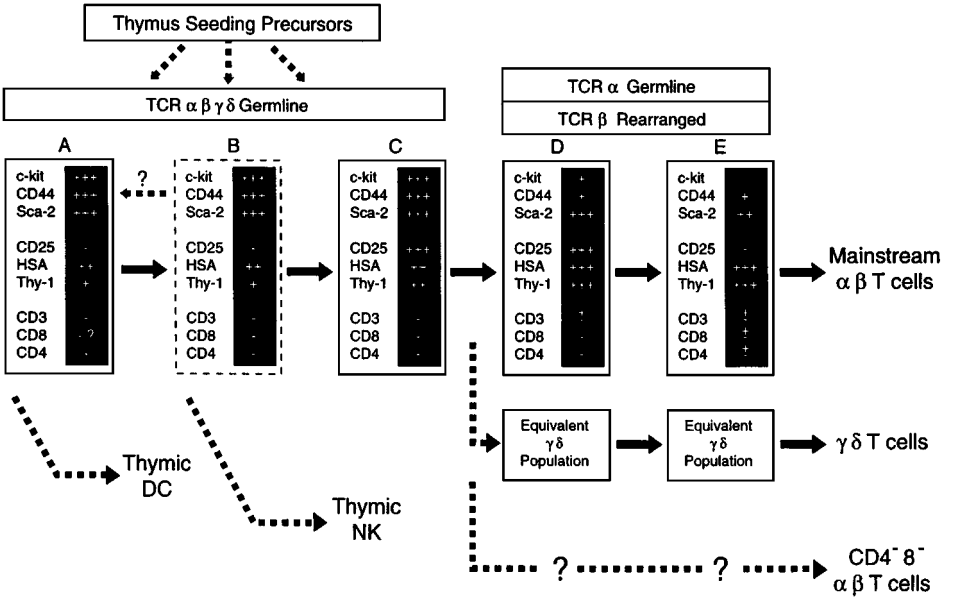

Figure 1 Populations of early T precursors in the mouse thymus. Only populations *A*, *B*, and *C*, together around 0.1% of all thymocytes, are early precursors without TCR gene rearrangement. In the adult thymus populations *A* and *C* are present, with a probable trace of *B*. In the early embryonic thymus population *B* is more prominent than population *A*. Arrows indicate developmental pathways, the solid ones probable, the broken ones possible.

$CD3^+4^-8^-$ TCR$\alpha\beta^+$ T cells, which can be produced from the $CD25^+$ subset on culture with IL-7 (43). The other is the lineage of $\gamma\delta$ T cells which develop in and exit from the adult thymus. These can be generated from $CD3^-4^-8^-$ cells that are $CD25^+$, as well as from those that are $CD25^-$ 44^{lo} HSA^{hi} Thy 1^{hi} (43, 44). Most of the $\gamma\delta$-expressing T cells in the adult thymus are proliferating HSA^{hi} Thy 1^{hi} cells, and those that exit the adult thymus also have this phenotype (47). As far as surface phenotype is concerned, these $\gamma\delta$ T cells therefore pass through developmental stages indistinguishable from populations *D* and *E* of the $\alpha\beta$ T lineage in Figure 1. An important issue, however, is whether these cells were precommitted to the $\gamma\delta$ lineage at a much earlier stage. Dudley et al (48) have recently reported that many $\alpha\beta$ T cells show evidence of failed attempts at δ and γ gene rearrangement, suggesting a very late and stochastically determined choice of lineage after rearrangement has commenced. Populations *D* and even *E* may therefore be not just similar but identical for the $\alpha\beta$ and $\gamma\delta$ lineages.

The $CD3^-4^-8^-25^+44^+$ c-kit$^+$ population of the adult mouse thymus (*C* of Figure 1) thus fulfils our criteria for an early T-precursor population. A different and apparently earlier T-precursor population (*A* of Figure 1) was first isolated in our laboratory (49), then by other groups (35, 43). This population expresses moderate levels of CD4, sufficient for the population to be lost when preparing $CD3^-4^-8^-$ thymocytes by depletion procedures, and it contains mRNA for CD4 (49). The population also shows low surface staining for CD8 (H Nakauchi, personal communication; L Wu, unpublished), but to date we have not found CD8α mRNA in these cells (J Ismaili and L Wu, unpublished). There is no obvious function for the premature CD4 expression by this so-called "low CD4" precursor, and it loses CD4 as it progresses to the $CD25^+$ stage. A similar population, but lacking CD4, is found in the embryonic thymus (see below) and can be detected by surface markers at trace levels in the adult (*B* of Figure 1). The "low CD4" precursor (*A* of Figure 1) has Cβ, Cγ, Vβ2, Dβ2 and Jβ2 genes in germline state (49; L Wu, unpublished), although it already expresses the recombinase activating genes RAG-1 and RAG-2 (50; L Wu, unpublished). The surface phenotype of this population is more akin to the bone marrow HSCs than later T-lineage precursors, being low for Thy 1 and high for Sca-1, CD44 and c-kit. However, it differs from bone marrow HSCs in expressing Sca-2. This surface molecule is a member of the Ly 6 family and is equivalent to MTS 35 or TSA-1 (51). Its function is unknown although antibodies against the molecule will disrupt $\alpha\beta$ T cell development in fetal thymus organ cultures (52).

Although these minute populations of early T precursors consisting of only 0.1% of thymocytes (*A*, *B* and *C* of Figure 1) are as close as has been obtained to the cells seeding the thymus from the bloodstream, they are probably still too numerous to be themselves these original immigrants. They are likely to

represent their expanded and possibly differentiated progeny. Nor is it clear that the cells seeding the thymus must progress via the *A* to *B* to *C* precursor sequence of Figure 1 en route to T cells, although this is the most logical progression. Entry could be at several points if the cells seeding the thymus themselves differ in maturation state. In addition, the "low CD4" precursor population could represent oligopotent precursors normally branching into a different lineage (see below), but recruited back into T cell development in the exceptional environment of the irradiated thymus used for T-precursor assays.

The phenotypic similarities of these early T precursors in the thymus to bone marrow HSCs prompts the question of their commitment to the T lineage. To date we have found no lineages other than $\alpha\beta$ and $\gamma\delta$ T cells being derived from the $CD3^-4^-8^-25^+$ population (D of Figure 1); this now needs checking by more sensitive procedures and with separate isolation of the earlier $CD44^{hi}$ c-kit^{hi} subset. However, the "low CD4" precursor population as a whole is clearly oligopotent. As well as forming $\alpha\beta$ and $\gamma\delta$ T cells on seeding the thymus, it produced typical small $B220^+$ s-Ig^+ B lymphocytes if injected intravenously (34). However, it formed very few spleen colonies and produced no detectable donor-derived myeloid cells (Mac-1^+ or Gr-1^+) when injected into Ly 5-disparate irradiated recipients, although a very low incidence of myeloid colonies was obtained in culture (34). Matsuzaki et al (35) have confirmed these findings, showing that the Lin^- Thy 1^{lo} c-kit^{hi} thymocytes are capable of forming as well as T cells, B cells, and $NK1.1^+$ cells (presumed NK cells). Thus, this population has many of the features expected of a common lymphoid-restricted precursor. It was therefore surprising when work in our laboratory revealed it also served as a precursor of DC on transfer to irradiated recipients, without concomitant formation of macrophages or granulocytes (53, 54). Although the yield of DC was low compared to T cells (1 per 2000), this is the normal ratio in the thymus, so all thymic DC could derive from the "low CD4" precursor population. Either some residual myeloid activity persists in the thymic precursor population, or a particular subclass of thymic DC is of lymphoid rather than myeloid origin.

Although this oligopotent, "low CD4" early thymus precursor population is remarkably homogeneous by surface marker analysis, there is no direct evidence that all these precursor activities derive from one cell, or even that most of the cells have T-precursor activity. Attempts to measure T-precursor frequency in this and other thymus populations (by limit-dilution repopulation of fetal thymus organ cultures) suggest a very low incidence of active cells (55). However, this may reflect a limited efficiency in seeding the right microenvironment or a requirement for limiting accessory cells. It is possible that the "low CD4" precursor population includes a variety of unipotent precursors of

identical surface phenotype, all derived from a multipotent form of HSC that seeds the thymus in low numbers, but then undergoes rapid differentiation and lineage restriction. The types of restricted precursors needed within the thymus might then be selectively expanded. New clonal assays are required to distinguish these various models.

Fetal Mouse Thymus

As discussed above, there is strong evidence that the embryonic thymus is seeded by and retains some multipotent stem cells (30, 31, 38), although this does not exclude the entry of T-committed precursors as well (38). Are the early intrathymic T precursors derived from these seeding cells different from those in the adult? With Antica & Scollay (56), we have sought an equivalent of the "low CD4" precursor (*A* of Figure 1) in the embryonic thymus. At day 14–15 of embryonic life no such population expressing significant levels of CD4 could be discerned, but a small population of $CD3^-4^-8^-25^-$ thymocytes expressing all the other markers (resembling *B* of Figure 1) was detected; this population gradually acquired CD4 until by day 18 it resembled the adult low CD4 precursor. However, in contrast to the adult precursor and to other precursors in the embryonic thymus, it proved very inefficient at repopulating an irradiated adult thymus (56). Hozumi et al (57) have isolated from the day-15 embryonic thymus a similar $CD3^-4^-8^-$ population that was $CD44^+$ c-kit$^+$ Thy 1^{lo} (resembling population *B*, Figure 1), and they have shown that, like the adult "low CD4 precursor", it has V, D and J β TCR genes in germline state, while already expressing RAG-2. However, they found that this population did have T-precursor activity, in a fetal thymus organ culture system. This suggests that these very early embryonic T precursors differ from those in the adult in requiring an embryonic environment for T-lineage development. This possibility now needs testing in side-by-side repopulation assays, using adult and embryonic precursors isolated in exactly the same way.

A further characteristic of embryonic thymus early T precursors, and of most thymocytes found at 14–16 days of embryonic age, is their expression of the low-affinity Fc receptors for IgG (FcγRII/III) (58). This is a characteristic marker of NK cells, although these thymocytes lack other NK markers and lack NK function. Such Fc receptors are not detected on adult thymus "low CD4 precursors" (L Wu, unpublished). Rodewald et al (58) have shown these early $CD3^-4^-8^-$ FcγRII/III$^+$ thymocytes are precursors of $\alpha\beta$ T cells when transferred into an irradiated thymus, but that they produce mature, functionally active NK cells when transferred intravenously. Likewise Brooks et al (59) have demonstrated that the majority of day-14 embryonic thymocytes can develop into mature NK cells in cultures containing IL-2. Thus there is strong evidence, although not as yet a formal clonal proof, that in the embryo T and NK cells

have a common precursor. This capacity to form NK cells appears to be retained for some time by these embryonic thymus early T precursors, even if it is not used in the thymic environment.

Human Thymus

The lack of data on the TCR-gene rearrangement status of early human thymocyte subpopulations limits our ability to delineate the early T precursors. Until recently lack of a suitable thymus reconstitution assay was also a limitation, which the SCID-hu mouse system (26) and the development of human T precursors in human fetal thymus organ cultures (60) have now largely overcome. Deducing a developmental sequence by direct analogy with the murine pathway is complicated by the absence of CD25 as a clear signpost of the stage of TCR gene rearrangement, as in the mouse. However, there are plentiful surface markers in the human system, and more use has been made of intracellular markers such as TdT or cytoplasmic CD3 expression. Most of the developmental pathways proposed to date have been deduced rather than experimentally derived, by assuming a sequential loss of markers associated with bone marrow HSCs (e.g. CD34) and sequential gain first of early T cell markers (e.g. CD7, CD2, CD5, CD1), then of late T cell markers (CD4, CD8, CD3) (61–63). Some of these steps have now been verified by testing precursor-product relationships either in culture or in the new thymus reconstitution systems.

The earliest intrathymic T precursors in the human thymus would be expected to express CD34, found on HSCs and most early precursors. Indeed a proportion of $CD3^-4^-8^-$ thymocytes are $CD34^+$, and only these have effective T-precursor activity (64). It has been argued that the T-lineage marker CD7 appears even before thymus seeding, since some bone marrow and fetal liver cells are $CD34^+$ $CD7^+$; however, many of these may be developing into NK cells (65). Nevertheless, the likely earliest human thymocytes are $CD34^+7^+$, and the majority of these express cytoplasmic CD3, indicating T-lineage orientation (61–63). CD2 is also present on the earliest putative human thymus T precursors, although at reduced levels (61–63). Although CD5 also is claimed to appear very early (62), a population of $CD34^+$ $CD5^-$ precursors has been described (66); CD5 expression may mark a downstream precursor. CD1 expression marks a still later developmental step (61–63). As in the murine thymus, the early precursors express CD44 (33, 61–63). However, the earliest detectable putative precursors in the infant or even in the young adult human thymus do not express CD4 (63), in contrast to the postnatal and adult mouse, but as in the early mouse embryo. Overall the earliest T precursors in the human thymus probably have the surface phenotype $CD3^-4^-8^-$ $34^+44^+7^+2^{lo}5^-1^-$.

The human thymus, both pre and postnatal, contains precursor cells within the $CD3^-4^-8^-$ fraction able to generate lineages other than T cells (32, 33, 66).

The central question is whether these share a common precursor with T cells, other than a multipotent HSC. NK cells have been shown to develop from $CD3^-4^-8^-$ thymocytes, and the presence of CD3ϵ and δ proteins within fetal NK cells strongly supports a common T/NK precursor (67). However, Sanchez et al (66) found that the majority of NK precursors and NK cells in the $CD3^-4^-8^-$ fraction are $CD34^-56^+5^-$ and without T-precursor function. Nevertheless, an earlier common T/NK precursor may well exist. The myeloid potential of $CD3^-4^-8^-$ thymocytes (30) has been reexamined recently by Marquez and colleagues (33). By following the growth and differentiation of $CD34^+3^-4^-8^-$ thymocytes cultured in the presence of IL-7, they observed the simultaneous development of T-lineage cells (small $CD4^+8^+7^+44^-$ DR^-) and non-T cells consisting of both monocytes and DC (large $CD4^+8^-7^-44^+$ DR^+). Both pathways progressed via $CD1^+4^+$ intermediate stages, but these intermediates were separable by CD44 expression. This recalls the generation of both T cells and DC from the adult mouse low CD4 precursor (53). However, these cultures may have included two independent developmental sequences, derived from separate $CD34^+$ precursors which happen to have surface antigen features in common. Once again a clonal approach will finally be needed to determine if the early T precursors also have DC or myeloid developmental potential.

Extrathymic T Cell Development

A significant proportion of all T cells, especially those found between the epithelial cells of the gut mucosa, do not develop within the thymus. These have been well reviewed recently (68) and are not considered in detail here. Some of the T-lineage precursors found in bone marrow or in blood may be destined for such extrathymic development, rather than for thymus seeding. Although there is evidence for TCR-gene rearrangement within the gut epithelium itself, neither the major sites of extrathymic T cell expansion and differentiation nor the nature of the precursor cells involved is clear at present. This is an area demanding further investigation.

THE CONTROL OF EARLY T-PRECURSOR DEVELOPMENT

Although it is clear from the preceding survey that our ability to identify and order in sequence the earliest steps in T cell development is still limited, enough is known to begin asking how the processes are controlled. These multiple steps in T cell development are determined by changes in the expression patterns of lineage-specific genes and, downstream from the area of this review, by sequential rearrangement of TCR genes. These in turn are known to be controlled

at the DNA level by certain transcription factors, controlled in the cytoplasm by signal transduction molecules including a number of tyrosine kinases, and controlled at the cell surface by receptors for cell interaction molecules and cytokines. We consider recent progress only in those regulatory molecules that clearly have a selective impact on early stages of the T lineage.

Cytokines and Cytokine Receptors

Of the cytokines that have been proposed to control early T cell development, only IL-7 has so far been shown to have an essential role. IL-7 stimulates the proliferation of immature thymocytes and mature T cells, as well as B-cell progenitors, through the interaction with its high-affinity receptor (IL-7R) (69, 70). Its action on $CD3^-4^-8^-$ thymic precursors may be more to maintain viability and function than directly to induce proliferation (71). It promotes rearrangement of TCR β and γ chain genes in fetal thymus and liver (72, 73), although it is not clear whether this is a direct effect or a consequence of increased survival and proliferation. Recent studies by Peschon et al (74) on mutant mice genetically deficient in the IL-7R have revealed a profound reduction in thymic and peripheral lymphoid cellularity. Both T cell and B cell development are blocked at an early stage. Thymocyte development is blocked, although not completely, within the $CD3^-4^-8^-$ population prior to acquisition of CD25 and prior to TCR β chain gene rearrangement, possibly at the "low CD4" precursor stage. Since in the normal thymus an early expansion of precursors precedes acquisition of CD25 and TCR-gene rearrangement, this early T-precursor expansion may be the process dependent on signals from the IL-7R. Whether IL-7R functions alone or in concert with other cytokines remains to be established.

Nonreceptor Protein Kinases

The nonreceptor protein tyrosine kinase $p50^{csk}$, predominantly expressed in adult mouse thymus and spleen, functions as a regulator of signal transduction via src-family kinases including $p56^{lck}$ and $p59^{fyn}$ (75, 76). Since $p56^{lck}$ and $p59^{fyn}$ play important roles in lymphocyte development (77, 78), $p50^{csk}$ may contribute to these regulatory circuits. Recent studies by Gross et al (79) on gene targeting of $p50^{csk}$ have shown that although $csk^{-/-}$ progenitors colonize the developing thymus, T cell differentiation is blocked at an early stage, as is that of B cells. $Csk^{-/-}$ ES cells fail to develop into adult lymphocytes in chimeric mice, whereas they are able to support the development of other hemopoietic lineages (79). Therefore, the expression of $p50^{csk}$ is selectively required for the maturation of early lymphoid progenitors, although it is not clear whether this regulatory function is via modulation of $p56^{lck}$ or via a different circuit. Nor is it clear whether $p50^{csk}$ acts before or after the stage of TCR β-gene rearrangement.

Disruption of Normal Development by a CD3ϵ Transgene

Another system for disrupting the normal control of early T cell development has been reported by Wang et al (80), who have introduced a human CD3ϵ transgene into mice. Mice with a high number of transgene inserts had no mature T cells or NK cells but do have normal B cells. Thymocyte development was blocked at the $CD3^-4^-8^-44^+25^-$ stage, clearly earlier than with the recombinase deficient mice, either RAG-1 null (81) or RAG-2 null (82), where NK cells develop normally. However, with lower copy numbers of the transgene, thymocyte development was blocked at the later $CD3^-4^-8^-44^-25^+$ stage, and normal NK cells were then detected. These experiments add further support to the notion that T cells and NK cells develop from a common precursor, although separate precursors with a common developmental mechanism involving the CD3ϵ protein is another interpretation. The data indicate that cytoplasmic tail of the CD3ϵ protein is needed to disrupt development. Whether this occurs by a positive signal or because of competitive binding of an essential signal transduction molecule is yet to be determined.

Transcription Factors

Of all the transcription factors proposed to have a role in T cell differentiation only one, the Ikaros protein, has been found selective for lymphoid development. Ikaros, a zinc-finger DNA binding protein, is comprised of five zinc finger modules organized in two clusters. It was cloned on the basis of its ability to bind to the enhancer of a gene encoding an early T cell differentiation antigen, CD3δ (83, 84). During development, Ikaros mRNA is first detected in the mouse fetal liver and then in the embryonic thymus, when hemopoietic and lymphoid progenitors initially colonize these organs. In adult mice Ikaros is expressed in T cells and their progenitors as well as in early B cells (83). In addition to this restricted expression pattern of Ikaros mRNA, high affinity binding sites for the Ikaros protein can be identified in the regulatory domain of many lymphocyte-specific genes including genes coding members of the CD3-TCR complex, CD4, CD2, TdT, IL-2Rα, NF-κB, Ig heavy and light chains, and the early B cell differentiation antigen mb-1 (84). Thus, Ikaros seemed likely to be a major determinant of lymphoid lineage commitment. This was confirmed in the recent study of Georgopoulos et al (85), which demonstrated that mice homozygous for a germline mutation in the Ikaros DNA-binding domain lack both mature and immature T and B lymphocytes as well as NK cells. In contrast, the erythroid and myeloid lineages are intact or elevated in these mutant mice. The developmental block is at an earlier stage than in recombinase-deficient mice (81, 82), and perhaps still earlier than in the CD3ϵ transgenic mice (80). However, it is not yet clear whether the Ikaros gene

products control the commitment of HSCs to a common lymphoid progenitor, or whether they function on separate restricted lymphoid-lineage progenitors that utilize the same mechanism in their subsequent development. Studies on the developmental potential of HSCs isolated from the Ikaros mutant mice, and on the genetic elements downstream of Ikaros gene, should provide more insight into the early steps in commitment to the lymphoid lineage.

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IMMUNOTOXINS: An Update

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ABSTRACT

The use of immunotoxins (ITs) in the therapy of cancer, graft-vs-host disease (GvHD), autoimmune diseases, and AIDS has been ongoing for the past two decades. ITs contain a targeting moiety for delivery and a toxic moiety for cytotoxicity. Theoretically, one molecule of a toxin, routed to the appropriate cellular compartment, will be lethal to a cell. Newly developed MoAbs, toxins, and molecular biological technologies have enabled researchers to construct ITs that can effectively kill many different cell types. In fact, phase I/II clinical trials have given promising results. Although nonspecific toxicity and immunogenicity still limit the use of IT therapy, these agents hold enormous promise in an optimal setting to treat minimal disease.

INTRODUCTION

For the past several decades, researchers and clinicians have been using drugs and/or radiation to kill tumor cells. While chemotherapy and radiotherapy continue to be the treatments of choice following surgery, or as front-line therapy when surgery is contraindicated, these therapies are only semiselective for malignant cells (1). In contrast, targeted drug therapy has the potential for

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greater specificity. With the advent of monoclonal antibody (MoAb)[1] technology, researchers have been able to target different agents to target cells more effectively. For example, immunotoxins (ITs) are cytotoxic agents that consist of a MoAb as the targeting moiety, a plant or bacterial toxin that has had its intrinsic binding domain removed or modified, and a linker that is stable in the circulation but labile inside the cell (2–5). Additionally, cytokines and soluble receptors are being used as targeting moieties (5, 6). The toxins are very powerful, and since they are catalytic (able to react with multiple intracellular targets), fewer than 10 molecules in the cytosol of a target cell are enough to be lethal (7, 8). Unlike chemotherapeutic agents, these toxins kill both resting and dividing cells. Hence, they have the potential for killing tumor cells that are not in cycle (i.e. dormant tumor cells) and are therefore spared by chemotherapy. In addition to their use in the therapy of cancer, ITs are also being developed to treat immunologically related diseases. For example, ITs can target T cell populations to prevent organ graft rejection and graft-versus-host disease (GvHD), to ameliorate symptoms of autoimmune diseases, or to target HIV-infected T cells.

Despite the conceptual simplicity of ITs, avoidance of nonspecific toxicity as well as efficient internalization and intracellular trafficking of these agents are just a few factors that must be considered in constructing optimal ITs. Nevertheless, the results thus far achieved in early clinical trials have been encouraging, and there is little doubt that some form of targeted therapy will eventually be used clinically.

BIOCHEMISTRY OF IMMUNOTOXINS

Targeting Moiety

The targeting agents currently used to construct ITs are MoAbs, growth factors/cytokines, and soluble receptors (9–12). MoAbs are the most frequently used targeting agents. In the case of tumor cells, MoAbs that recognize differentiation antigens (Ags) on normal cells are often used because unique tumor-associated Ags have not been identified for most human tumors. Since tumor

[1]ABBREVIATIONS: Ab, antibody; Ag(s), antigen(s); BsAb, bispecific antibody; BLR, blocked ricin; (A)BMT, (autologous) bone marrow transplant: dgA, deglycosylated ricin A-chain; DT, diptheria toxin; EAMG, experimental autoimmune myasthenia gravis; EC(s), endothelial cell(s); EF-2, elongation factor 2; ER, endoplasmic reticulum; FGF-1, fibroblastic growth factor-1; GvHD, graft-versus-host disease; HLA, human leukocyte antigen; Ig, immunoglobulin; IDDM, insulin dependent diabetes mellitus; IT(s), immunotoxin(s); IFN-γ, interferon-γ; IL, interleukin; IL-2R, interleukin 2 receptor; MHC, major histocompatability complex; MoAb, monoclonal antibody; MTD, maximum tolerated dose; PAP, pokeweed antiviral protein; PE, Pseudomonas exotoxin; RA, rheumatoid arthritis; RIP(s), ribosome inactivating protein(s); RTA, ricin toxin A-chain; SAP, saporin; TGF-α, transforming growth factor-alpha; VLS, vascular leak syndrome.

cells often express higher levels of these differentiation Ags, the tumor cells may be preferentially killed (13, 14). One drawback in using MoAbs as the targeting agent is that they are usually of mouse origin and are therefore immunogenic in humans. If prior treatment or the disease itself is immunosuppressive (e.g. B cell lymphoma or AIDS), this is less problematic. In cases where the patient is not immunosuppressed, the use of less immunogenic chimeric or humanized Abs is being explored (15–21). Alternatively, recombinant growth factors (specifically cytokines) or soluble receptors (e.g. CD4) can be less immunogenic. However, host-derived cytokines or cytokine receptors present in the serum may compete with ITs and limit their effectiveness in vivo.

Cytokines make effective targeting agents because their affinity for their ligands can be several orders of magnitude higher than that of typical antibodies (22). Cytokine receptors can then effectively internalize the cytokine-based IT via receptor-mediated endocytosis (23, 24). Furthermore, cytokine receptors are often modulated during cell activation or differentiation, making it possible to target a specific cell population selectively [i.e. ITs can be targeted to certain growth factor receptors that are often overexpressed on tumor cells (13)]. Care must be taken to ensure that the growth factor portion of the IT does not retain some of its agonistic effects, which could promote proliferation of the targeted cell population. Such effects, although transient or dose-dependent, have been observed with ITs targeting IL-2, IL-4, and IL-6 receptors (25–27).

Bispecific antibodies (BsAb) are novel targeting agents constructed by linking either chemically or genetically two different Fab fragments, one arm of which is directed against a target cell and the other against effector T cells or NK cells (e.g. anti-CD22/anti-CD3-RTA)(28–31). In one case, a BsAb could deliver both a toxin and an effector cell to the site of a tumor because the arm directed against the effector cell did not kill it (31). BsAb ITs recognizing two antigens on the same cell have also been constructed (29, 30). For example, anti-CD4 antibodies have been linked to anti-CD26 or anti-CD29 in attempts to modulate GvHD by targeting cells that express both antigens. These ITs target and kill activated helper/inducer (memory) T cell subsets, because, for killing to occur, the BsAb-IT must bind simultaneously to both determinants on a cell; univalent binding does not induce internalization (29, 30). Toxins have also been delivered to cells by constructing BsAb ITs in which one arm recognizes the tumor cell (e.g. anti-CD22) and the other recognizes a toxin (e.g. anti-saporin) (32).

Toxin Moiety

The most commonly used toxic moieties for making ITs are derived from either bacteria [e.g. *Pseudomonas* exotoxin (PE) or diphtheria toxin (DT)], or plants (e.g. ricin or abrin) (Table 1). Both types of toxins kill cells by inhibiting protein

Table 1 Toxins used for the preparation of ITs

PLANT TOXINS (N-glycosidase for 28S rRNA)
Two chain toxins
1. Abrin
2. Ricin
3. Modeccin
4. Viscumin
Single chain toxins (RIPs)
1. Pokeweed anti-viral protein (PAP)
2. Saporin (SAP)
3. Gelonin
4. Momoridin
5. Trichosanthin
6. Barley toxin
BACTERIAL TOXINS (ADP Ribosylation of EF-2)
1. Diptheria toxin (DT)
2. *Pseudomonas* exotoxin (PE)
FUNGAL TOXINS (single chain RIPs; ribonuclease of 28S rRNA)
1. α-sarcin
2. restrictocin

synthesis (Figure 1) (33, 34). For example, PE and DT inactivate elongation factor 2 (EF-2), and ricin and abrin inactivate the EF-2-binding site on the 28S ribosomal subunit (35). Most plant and bacterial toxins are synthesized as single-chain proteins which are then processed posttranslationally into two polypeptide chains linked together by interchain disulfide bonds (33, 36, 37). In most cases, there are at least two (and sometimes three) functional domains. One domain contains the enzymatic activity (A-chain), while the other contains the targeting/binding activity (B-chain) (33, 38). Usually the binding domain is deleted and replaced by a specific ligand. Alternatively, ITs can be constructed with the entire toxin (holotoxin), but in this case, the lectin-binding sites must be blocked (39). A third domain may also be involved in the translocation of toxin into the cytoplasm (e.g. PE). Different domains have been manipulated and/or deleted by biochemical or genetic modifications to generate more effective and less immunogenic ITs.

Ribosome inactivating proteins (RIPs) are a subgroup of the plant toxins [e.g. pokeweed antiviral protein (PAP), saporin (SAP), or gelonin—see Table 1] (40–42). They are synthesized as single-chain proteins that lack a targeting moiety (or B-chain equivalent) (41, 43, 44). These proteins are functionally analogous to the A-chain of ricin or abrin in that they inhibit protein synthesis by inactivating the 28S RNA (41). Since the RIPs lack a B-chain equivalent,

they must be linked to a cell-binding ligand in order to be cytotoxic. The advantage of using RIPs is that rigorous purification to eliminate the B-chain is not necessary.

ITs have also been constructed using hybrids of both plant and bacterial toxins (e.g. ricin A-chain and the A fragment of DT) (45). Because bacterial and plant toxins act at different steps in protein translation, a combination of their activities could be more effective. In fact, some ITs constructed with these hybrid toxins are 100–1000-fold more effective in inhibiting tumor cell growth than are ITs prepared with either toxin alone (45).

Linkers

For in vivo therapy, the toxic moiety of the IT must be coupled to the targeting ligand so that it remains stable in the blood and tissues but is labile within the target cell. The A-chain has to be separated from the targeting domain (e.g. MoAb or B-chain in the case of holotoxin) for effective translocation into the cytoplasm. A variety of cross-linkers have been developed based on whether the holotoxin or the active domain of the toxin (or RIP) is used (46).

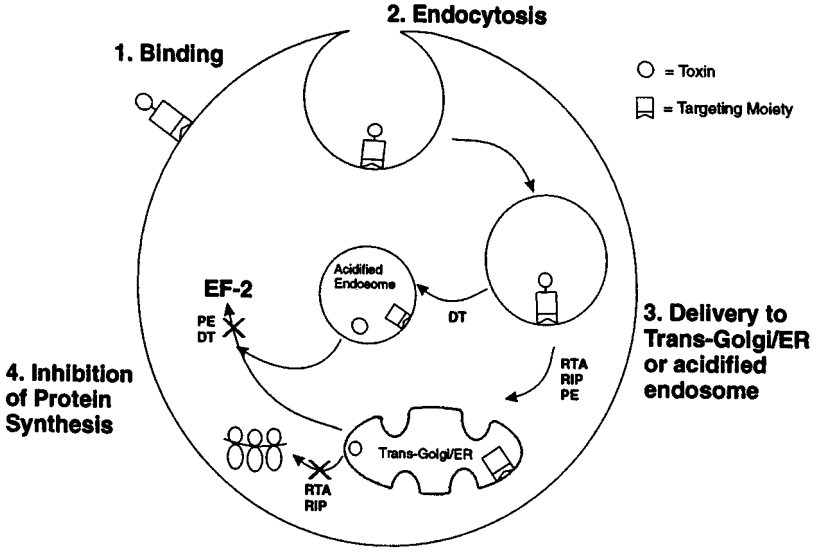

Figure 1 Mode of action of ITs containing RTA, RIPs, PE, or DT. ITs bind to the cell surface via the targeting moiety and are internalized by receptor-mediated endocytosis. The plant toxins and PE are routed to the *trans*-golgi network, and DT is routed to an acidified endosome. In these compartments, the toxins are separated from the targeting moiety. The toxins kill cells by inhibiting protein synthesis; the plant toxins damage 28S rRNA, and the bacterial toxins inactivate EF-2.

In the case of holotoxins, a naturally occurring disulfide bond exists between the catalytic domain and the remainder of the toxin that would normally be reduced once inside the cell. Therefore, when preparing ITs, holotoxins are linked to a targeting moiety via a thioether linkage that is highly stable and nonreducible (1, 47). Unlike holotoxin-containing ITs, ITs containing A-chains or RIPs must introduce a disulfide bond between the targeting ligand and the toxin in order to be active (1, 47). Heterobifunctional cross-linkers introduce an activated thiol group into the ligand but this linkage is labile to reduction and the resulting ITs are not as stable or as long lived in vivo (46, 48–50). Alternatively, fusion toxins have been prepared by fusing and expressing the genes for the toxin and targeting ligand (5, 51). When toxins such as PE or DT are used, the fusion protein will contain the proteolytic cleavage site that is normally present in the native toxin. The enzymatic domain can therefore be separated from the targeting moiety by proteolysis once the IT is internalized. When a plant toxin is used (which has no cleavage sites in its native sequence), nucleotides that encode a cleavage site must be introduced between the targeting and toxic moieties (53).

FACTORS AFFECTING THE POTENCY OF AN IT

Many factors are critical in determining the cytotoxicity of an IT. These include the binding affinity of the targeting moiety and the density of the target Ag on the cell (54, 55). Ligands that bind with low affinity, or bind to Ags whose density on the surface is low, often require up to 10^4 times more IT to kill cells (50). Furthermore, the target Ag must be naturally internalized or induced to do so (57). When MoAbs are used as the targeting agents, the epitope on the target Ag recognized by the IT may also affect the potency of an IT (55, 58–60). For example, MoAbs that bind to epitopes that are relatively close to the membrane make better ITs than those that bind to sites that are more distal. It has been speculated that proximity of the target epitope to the membrane facilitates the translocation of the toxic portion of the IT across the membrane into the cytosol (60). Because only 5–25% of MoAbs make potent ITs, many MoAbs must be screened to select those that make effective ITs (61).

Once an IT is internalized, its intracellular routing is one of the most critical variables in determining its potency (57, 58, 62). The intracellular routing pathways for many of the toxins have not been defined in great detail. In the case of PE, mutational analysis indicates that the carboxy-terminal sequence Arg-Glu-Asp-Leu-Lys (REDLK) is important for its cytotoxic action (63). While removal of this sequence does not affect its ADP-ribosylating activity, its lack of cytotoxicity, suggests that this sequence may be involved in either the translocation of toxin into the cytosol, or in its routing to an

intracellular compartment from which translocation can take place. This sequence resembles the well-defined endoplasmic reticulum (ER) retention signal Lys-Asp-Glu-Leu (KDEL) (64–66). In fact, when the carboxy-terminal sequence of PE is changed to KDEL, the molecule remains fully cytotoxic. The addition of a KDEL peptide sequence to the A-chain of ricin toxin results in increased cytotoxicity when the mutant A-chain is reassociated with its native B-chain (67). Unlike PE, native ricin does not contain a natural KDEL-like sequence.

The intracellular compartment into which the IT is routed will ultimately determine whether the final translocation step into the cytosol takes place rapidly and efficiently. Unfortunately, once internalized, most of the IT is routed to the lysosomes, where the toxin is degraded. Lysosomotropic amines (e.g. NH_4Cl and chloroquine) and carboxylic ionophores (e.g. monensin), which increase the pH of the intracellular compartments, protect cells from PE and DT but increase their sensitivity to the plant toxins ricin and abrin (68–71). Possibly, therefore, translocation of the plant toxins into the cytosol takes place in the Golgi complex (71, 72). In support of this hypothesis, alterations in the Golgi apparatus sensitize cells to ricin (68, 69, 73, 74).

In several rodent tumor models, the inability of a single type of IT to cure the animals is due to the survival and subsequent outgrowth of IT-resistant tumor cells. These mutants may lack the target Ag entirely or express it at levels too low for effective IT-mediated killing. Alternatively, the cell may express the target Ag but have a defect in internalization and/or intracellular routing. In some cases, such mutant cells can be eradicated with cocktails of two or more ITs recognizing different target Ags, provided that any defect in the intracellular trafficking is not common to both Ag-entry pathways.

TARGETING ITS TO B AND T CELLS

ITs have been used to treat numerous immunologically related diseases in both animals and humans (4, 9, 75–80). For example, ITs have been used to kill hematopoietic tumors (e.g. lymphoma), treat autoimmune diseases [e.g. rheumatoid arthritis (RA) and diabetes], prevent GvHD, and kill HIV infected T cells. Clinical responses have been excellent (28% complete responses) when accessible circulating T cells have been targeted as a treatment for steroid-resistant GvHD or autoimmune diseases (81–84). In the case of B and T cell tumors, lineage-specific differentiation Ags can be targeted since normal B and T cells can be repopulated from stem cells (Tables 2 and 3). Because tumor cells often express higher levels of these Ags, they will be preferentially killed.

Cytokine-based ITs have also been used to target B and T cells (Table 3). For example, numerous IT constructs target the IL-2 receptor (IL-2R), which exists

Table 2 MoAb-based ITs used to target cells of the immune system

Antibody against	Disease Indication	Targeted Cell	References
CD2	GvHD	T cell	(138)
CD3	GvHD, Diabetes	T cell	(81, 106, 138, 139)
CD4	HIV	T cell	(114–116, 140)
CD5	GvHD, RA	T cell, B cell	(4, 138, 140, 141)
CD7	GvHD, non-Hodgkin's lymphoma (ABMT)	T cell	(4, 138)
CD11a	GvHD	T cell, NK cell	(138)
CD18	GvHD	T cell	(138)
CD19	Non-Hodgkin's lymphoma, ABMT	B cell	(4, 140, 141)
CD22	Non-Hodgkin's lymphoma	B cell	(4, 140, 141)
CD25	GvHD, non-Hodgkin's lymphoma (ABMT),	Activated T cell	(113, 122, 123, 140)
CD30	Hodgkin's lymphoma	Reed-Sternberg cell	(4, 141)
CD33	Myeloid leukemias	Myeloid lineage	(111)
Vβ6TCR	EAMG	T cell	(142)

Table 3 Growth factor/cytokine based ITs

Targeted structure	Immunotoxin	Disease Indication	References
IL-2R	DAB_{486}-IL-2, DAB_{389}-IL-2, IL-2-PE40, IL-2-PE664glu, IL-2-DT	Lymphoma, AIDS, autoimmune disease	(6, 22, 23, 140, 143)
IL-4R	IL-4-PE40	Lymphoma, various tumors	(100, 140)
IL-6R	DAB_{389}-IL-6, IL-6-PE40	Myeloma, hepatoma, Kaposi sarcoma	(99, 105, 140, 144)
IL-7R	DAB_{389}-IL-7	Lymphoma, leukemia	(101)
IL-9R	IL9-ETA	Hodgkin's disease, lymphoma	(102)
IL-15R	DAB_{389}-IL-15	Lymphoma, leukemia	(103)
EGF-R	PE40-TGFα, TP40 DAB_{389}-EGF	Various tumors	(14, 140, 145)
FGF-R	acidic FGF-PE, basic FGF-SAP	Limb-threatening ischemia	(128, 129)
Melanotropin receptor	DAB_{389}-MSH	Melanoma	(24, 146)
GM-CSF-R	GM-CSF-SAP, DT-GM-CSF	Lymphoma, leukemia	(104, 105)

as a complex of the 55-kDa α-chain (p55 or CD25) and the 75 kDa β-chain (p75 or CD122) (85–87). There is also a γ chain (p64) that associates with the β-chain of this complex for signal transduction, but it does not bind IL-2. While the low-affinity IL-2R (p70) is expressed on all T cells, the high-affinity receptor (p70, p55) is expressed only transiently on activated lymphocytes (88). Since the high-affinity IL-2R is not expressed on nonlymphoid cells, it is theoretically possible to target this cell population selectively with low concentrations of cytokine fusion proteins. For example, recombinant forms of DT (e.g. DAB-IL2) and PE (e.g. PE40-IL2) are two chimeric ITs that target the high-affinity IL-2 receptor (11, 89–91). These ITs bind to the low-affinity IL-2R, so that relatively high concentrations are needed to kill cells. These and other cytokine-based ITs have been used to treat lymphoma, RA, and type I insulin-dependent diabetes mellitus (IDDM), and for prevention of graft rejection (92–97). Targeting of other lymphoid cells using recombinant fusion toxins has been achieved using IL-4, IL-6, IL-7, IL-9, IL-15, GM-CSF, and TGF-α as the targeting moiety (98–105).

GvHD in Bone Marrow Transplantation

GvHD is a major problem when allogeneic bone marrow transplants (BMT) are used to rescue patients with leukemias and lymphomas who have been treated with supralethal doses of chemotherapy (12). Because donor T cells in BMT mediate GvHD, patients are immunosuppressed with steroids when undergoing BMT. T cell–specific ITs are being used in clinical trials to overcome this problem (81, 106). IT therapy for GvHD is most effective when IT is administered after the BMT but before GvHD develops. MoAbs or cytokines can be used to target specific surface antigens on T cells (e.g. CD5, CD3, IL-2) (81, 106). In some cases, these surface antigens may also be on other lymphoid cells (e.g. $CD5^+$ B cells), but IT-mediated killing of these cells is generally well tolerated.

There is a reduced incidence of GvHD when anti-CD5-based ITs are used in patients who receive an HLA-matched BMT but from an unrelated donor (107). Side effects commonly observed during IT therapy [e.g. vascular leak syndrome, (VLS)] were also observed in these patients, although the effects were tolerable at effective doses. However, some patients were susceptible to viral infections. Therefore, while the ITs were effective in preventing GvHD, rapid efforts must be made to restore the immune system to prevent lethal viral infections (e.g. by coadministration of IL-3). BMT patients who received bone marrow from HLA unmatched and unrelated donors have also been treated with anti-CD5 based ITs, but in combination with other immunosuppressive drugs (108, 109). This treatment was not effective in preventing GvHD.

Bone Marrow Purging in Autologous Bone Marrow Transplantation

ITs are being used to purge bone marrow of tumor cells in cases where cancer patients undergo autologous BMTs (111). Bone marrow from these patients is removed and stored prior to systemic treatment with supralethal doses of chemo- or radiotherapy. The bone marrow progenitors are then reinfused back into the patient.

Bone marrow purging has also been used to remove malignant B cells and T cells from autologous bone marrow. In one clinical trial, leukemic T cells were removed by incubating the bone marrow with ITs directed against CD5 and CD7 (112). This treatment was effective in some patients. In those patients in whom relapses did occur, the relapses were due to the treatment failure rather than inefficient purgings of the marrow. This ex vivo procedure avoids the side effects and nonspecific toxicity observed when using ITs in vivo.

Autoimmune Disease

The targeting of T cells with ITs can also be used to treat autoimmune diseases such as RA, IDDM, systemic lupus erythematosus, and experimental allergic encephalomyelitis, since autoreactive T cells have been implicated in the pathogenesis of these diseases (83, 93, 113). In Phase I clinical trials, patients with RA have been treated with ITs that target $CD5^+$ T cells (83). A 50% overall decrease in joint pain and swelling was observed. In a Phase II clinical trial using anti-CD5 IT to treat RA, a high initial response rate was seen after 1 month of treatment and this was sustained up to 6 months in up to 25% of the patients (83). ITs that target additional surface Ags on T cells such as the IL-2 receptor (IL-2R) have resulted in similar and sometimes even greater reductions of symptoms in patients with RA (113). Patients with autoimmune IDDM have also been treated with ITs in clinical trials that target both CD5 or IL-2 (84). Preliminary studies indicate that both therapies decrease the symptoms seen in these patients.

HIV Infection and AIDS

ITs have been evaluated for the therapy of HIV infection. For example, ITs have been targeted to CD4, the cellular receptor for the virus (114–117). These ITs selectively destroy HIV^+ $CD4^+$ cells, but not HIV-$CD4^+$ cells, since the former are more sensitive to anti-CD4-targeted toxins (114–117). Soluble CD4 has also been conjugated to both RTA and PE, and in both cases has inhibited synthesis of viral proteins in infected cells as well as the spread of virus (114, 116). However, in the presence of anti-gp120 Abs, commonly found in the serum of AIDS patients, these ITs are less effective (118, 119). ITs have also been targeted to the HIV envelope protein gp160, as well as to its components

gp120 and gp41 (118–121). Unfortunately, ITs containing MoAbs directed against the immunodominant hypervariable region of gp120 do not kill cells infected with all strains of HIV. In fact, these ITs don't even kill the cells infected by all the HIV variants within the same patient. Although gp41 can be used as a target for ITs because it is more highly conserved (119), anti-gp41 Abs in the patients may reduce the effectiveness of these ITs (118–120).

ITs can be targeted to cellular receptors on normal activated T cells and monocytes (122, 123). For example, the IL-2R α-chain (CD25) is expressed on activated, virus-producing cells (122, 123), and ITs targeted to this receptor efficiently prevent viral replication. Fortunately, $CD25^+$ cells are rapidly replenished from the resting T cell pool so that significant toxicity to the patient should not result. However, while anti-CD25 ITs will kill normal cells expressing IL-2R, they do not kill latently infected cells. Targeting $IL\text{-}2R^+$ cells may also result in the killing of activated $CD8^+$ cells, which might be necessary to suppress the growth of HIV-infected cells (122).

Clinical Trials

Experimental studies suggest that ITs have optimal efficacy when administered in a single short course of therapy to patients with minimal, dormant, or premalignant disease (4, 78–80). However, Phase I clinical trials are designed to test the safety of a drug and require that patients have intractable disease with large bulky tumors. When the side effects, immunogenicity, and pharmacokinetics have been established and the maximum tolerated dose (MTD) has been determined, the IT can be used in Phase II clinical trials in patients with less advanced disease. In Phase II trials, efficacy is determined and a response rate of 20–40% (partial or complete remission) must be achieved in order for the drug to continue into Phase III clinical trials. To date, most ITs are just entering Phase II/III trials, and their efficacy, although it appears encouraging, is still under evaluation (4). Several observations have emerged from these Phase I/II clinical trials.

In eight separate IT trials, the number of patients achieving partial or complete remissions from cancer ranged from 12% to 75%. This is excellent for Phase I trials. To put this in perspective, more than 90% of chemotherapeutic drugs used today produced less than a 5% partial or complete remission in Phase I clinical trials. Patients with large solid tumor masses, however, rarely respond well to IT therapy because of the inaccessibility of the cells to the ITs (124).

In order to treat solid tumors more effectively with ITs, it has been proposed that an IT should be targeted to the dividing vascular endothelial cells (ECs), rather than to the tumor cells themselves (125). In this way, tumor cell heterogeneity will not be a problem and one can kill the tumor by starving the cells of necessary nutrients and oxygen (126). ECs are accessible, and because they are normal cells, their Ags are unlikely to mutate. This approach should be useful

Table 4 Summary of clinical trials

Disease	Immunotoxin	Toxicity	Clinical response	Reference
Metastatic Melanoma	Xomazyme-Mel	VLS, myalgia	1/22 CR, 9/22 mixed or stabilized	(147)
	Xomazyme-Mel	VLS	3/43 PR, 1/43 MR, 9/43 stabilization	(148)
	Xomazyme-Mel plus cyclophosphamide	VLS	4/20 PR	(149)
	Xomazyme-Mel plus cyclosporine	Arthralgia, SGOT/SGPT elevations, VLS	1/8 PR, 1/8 stable mediastinal disease	(150)
Colorectal Carcinoma Metastatic Breast carcinoma	Anti-gp 72-RTA	VLS, aphasia	5/16 mixed tumor regressions	(151)
	260F9-RTA (bolus)	VLS, myalgia paresthesia	1/4 resolution of lung nodule	(152)
	260F9-RTA (continuous infusion)	VLS, myalgia, neuropathies	0/5	(153)
Ovarian carcinoma	Anti-OVB3-PE	SGOT-SGPT elevations, abdominal pain, encephalopathy	0/23	(154)
Non-Hodgkin's lymphoma	Anti-CD19-BLR (bolus)	SGOT/SGPT elevations thrombocytopenia	1/25 CR, 2/25 PR, 10/25 mixed/transient	(155)
	Anti-CD19-BLR (continuous infusion)	SGOT/SGPT elevations thrombocytopenia edema	2/43 CR, 5/43 PR, 11/43 transient	(155)
	Anti-CD19-BLR (continuous infusion)	Increases in liver function tests, decreases in platelet counts, myalgia, edema, VLS	2/34 CR, 3/34 Pr, 11/34 transient	(156)
	Anti-CD19-BLR	Liver dysfunction, thrombocytopenia, VLS	2/11 PR, 3/11 minor response	(157)
	Fab' anti-CD22 (RFB4) dg-RTA	VLS, myalgia	5/13 PR	(158)
	Anti-CD22 (RFB4) dg-RTA	VLS, myalgia	1/24 CR, 6/24 PR	(159)
	DAB_{486}-IL-2	Hepatic transaminase elevation, fever, hypoalbuminemia	9/32 response	(6)
	DAB_{389}-IL-2		1/17CR, 2/17 PR	(160)

(Continued)

Table 4 (continued)

Disease	Immunotoxin	Toxicity	Clinical response	Reference
Hodgkin's disease	Anti-CD30-Saponin	Thrombocytopenia, SGOT/SGPT elevations, proteinuria	3/4 PR	(161)
	DAB_{486}-IL-2	Hepatic transaminase elevation, proteinuria, hypoalbuminemia	1/15 CR	(92)
	Anti-CD25-dgA	VLS	1/12 PR, 5/12 stable disease	(162)
B-Chronic Lymphocyte Leukemia	Anti-CD5 (T101)-RTA	Fever	4/5 transient decrease in circulating tumor cells	(163)
T Cell lymphoma	Anti-CD5 (H65)-RTA DAB_{389}IL-2	VLS, dyspnea Hepatic transminase elevation, fever, hypoalbuminemia	4/14 PR 1/17 CR, 2/17 PR	(164)
Psoriasis	DAB_{389}-IL-2		Up to 65% of patients with decrease in PASI score	(165)
Brain tumor	Anti-transferrin-CRM107 (DT)		8/15 PR	(166)
Small cell lung cancer	Anti-NKH1-BLR	VLS	1/19 PR	(167)
GvHD	Anti-CD5 (H65)-RTA	VLS, myalgia, hematuria, tremors	9/32 CR, 7/32 PR, 6/32 MR	(109)
	Anti-CD5-RTA (Xomazyme-CD5) plus methotrexate and cycloporine or prednisone	Nephrotoxicity, VLS	9/15 acute GvHD, 6/8 chronic GvHD, 4 survived 1.1–2 years after BMT	(109)
Arthritis	DAB_{486}-IL-2	Hepatic transminase elevation, fever	9/18 > 25–50% improvement of symptoms	(93)
	DAB_{486}-IL-2		5/13 > 30% improvement of symptoms	(6)
IDDM	DAB_{486}-IL-2	Hepatic transminase elevation, fever	9/18 decrease insulin requirement	(6)

for treating a variety of solid tumors. Vascular targeting is based on the premise that Ags expressed on dividing or activated ECs in the tumor vasculature are different from those found on resting ECs. Some tumor cells secrete cytokines [e.g. interferon gamma (IFN-γ) or fibroblastic growth factor (FGF-1)] which induce an increased expression of surface Ag on ECs in the tumor vasculature [e.g. class-II major histocompatibility complex (MHC) or FGF-1 receptor], which can then be targeted (127–129). The ultimate success of vascular targeting will depend upon defining unique Ags on dividing or activated but not resting ECs. Surface markers such as EN7/44, endoglin (CD105), endosialin, and E-9 Ag are possible candidates for vascular targeting (130).

Side effects of IT therapy are different from those of chemotherapy or radiotherapy. While ITs do not damage rapidly dividing normal tissues, DT-, PE-, and nondeglycosylated ricin-based ITs routinely cause hepatotoxicity, and all the ricin-based ITs cause reversible VLS and myalgias (131–133). Hepatotoxicity from ricin-based ITs is due to carbohydrate-mediated binding to liver cells, while in the case of PE and DT, the explanation is unknown. It is believed that VLS and myalgia may be related to the ability of ricin A-chain to bind nonspecifically and damage normal vascular ECs. Multiple courses of IT therapy have been tolerated in patients, indicating that toxicity is not cumulative.

Optimal regimens for administration of the ITs are currently being investigated. The half-life ($T_{1/2}$) of ITs have generally been shorter than would be predicted to induce an optimal therapeutic index and the MTD is usually inversely proportional to $T_{1/2}$. In most of the Phase I trials (except those conducted in immunosuppressed lymphoma patients), anti-IT Abs were generated (134–137). In some cases these Abs were neutralizing, but in all cases they decreased the $T_{1/2}$ of the IT in the blood. Nevertheless, there were meaningful responses even in the presence of such Abs. So far, attempts to decrease the Ab responses by the use of immunosuppressive drugs have been unsuccessful. A few general, but preliminary, conclusions can be made from the clinical trials (Table 4): 1. The majority of the Phase I clinical trials have established MTDs, and the therapeutic concentrations of IT were reached in the blood at doses close to the MTD. However, blood concentrations were not maintained at therapeutic levels between infusions, suggesting that continuous infusion might be desirable. 2. MTDs should take into account whether or not circulating tumor cells are present. 3. Poorly vascularized, bulky, solid tumors are not suitable for IT-based therapies. Circulating cells and well-vascularized lymphomas appear to be better targets. 4. Anti-IT antibodies were generated in most trials (except those conducted in immunosuppressed lymphoma patients). In some cases, these antibodies were neutralizing but in all cases they decreased the $T_{1/2}$ of the IT in the blood.

CONCLUSIONS

The clinical responses observed in many of the IT clinical trials for the treatment of cancer, autoimmune disease, and GvHD have been impressive. In fact, for the therapy of cancer, ITs have yielded higher response rates in Phase I/II trials than some of the drugs used today (when tested in similar trials). It is clear that the use of ITs should be further explored. The generation of new constructs, combinatorial therapy, and, in the case of cancer therapy, treatment of tumors that are amenable to IT-mediated killing (e.g. minimal residual disease) will eventually result in effective treatment protocols.

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CELLULAR INTERACTIONS IN THYMOCYTE DEVELOPMENT

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ABSTRACT

Interactions between stromal cells and thymocytes play a crucial role in T cell development. The thymic stroma is complex and consists of epithelial cells derived from the pharyngeal region during development, together with macrophages and dendritic cells of bone marrow origin. In addition, fibroblasts and matrix molecules permeate the whole framework. It is now apparent that these individual stromal components play specialized roles at different stages of T cell differentiation. Thus, at the early $CD4^-8^-$ stage of development, T cell precursors require fibroblast as well as epithelial cell interactions. Later, at the $CD4^+8^+$ stage, as well as providing low avidity TCR/MHC-peptide interactions, thymic epithelial cells have been shown to possess unique properties essential for positive selection. Dendritic cells, on the other hand, are probably efficient mediators of negative selection, but they may not be solely responsible for this activity.

Alongside the functional roles of stromal cells, considerable progress is being made in unraveling the nature of the signaling pathways involved in T cell development. Identification of the pre-T cell receptor (pre-TCR) and associated signaling molecules marks an important advance in understanding the mechanisms that control gene rearrangement and allelic exclusion. In addition, a better understanding of the signaling pathways that lead to positive selection on the one hand and negative selection on the other is beginning to emerge.

Many issues remain unresolved, and some are discussed in this review. What, for example, is the nature of the chemotactic factor(s) that attract stem cells to the

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thymus? What is the molecular basis of the essential interactions between early thymocytes and fibroblasts, and early thymocytes and epithelial cells? What is special about cortical epithelial cells in supporting positive selection? These and other issues are ripe for analysis and can now be approached using a combination of modern molecular and cellular techniques.

OVERVIEW

Brief Historical Background

The first definitive experiments demonstrating the crucial role of the thymus in the development of the immune system were carried out in the early 1960s. The exciting events of this time have been documented by Jacques Miller (1). Miller was investigating the role of the thymus in leukemogenesis when he performed experiments which showed that if mice are thymectomized within 1 or 2 days of birth, they have a marked deficiency of lymphocytes, fail to reject foreign skin grafts, and are susceptible to infections. By 1967, Miller & Osoba were able to produce an influential review (2) that provided a basis for much of our current understanding of the importance of the thymus in immune responses.

Perhaps surprisingly, the thymus was a topic of considerable interest long before this period, at a time when its function was a total enigma. Discussion centered mainly on the developmental origins of the organ. Are the epithelial cells, which make up a substantial component of the thymic stroma, derived from pharyngeal ectoderm and/or endoderm? What is the origin of thymus lymphocytes?

Origins of Thymic Stromal Cells

The origins of the epithelial cells are particularly relevant to current studies of thymic function. Contact with thymic epithelial cells is necessary for all stages of thymocyte maturation, including positive selection (see below). In the nude mouse, some thymic epithelial cells are present, but they fail to support T cell differentiation (3). A gene mutated in nude mice has recently been identified and shown to encode a winged helix transcription factor (*Whn*) expressed in skin and thymus (4). Based on a morphological analysis of murine thymic development in normal and nude mice, it was claimed that both ectoderm and endoderm normally contribute to thymic epithelium, but in nude mice the ectodermal component fails to develop (5). Thus failure of normal *Whn* expression might result in the absence of a crucial ectodermal contribution to the thymus (6). However, one of the few experimental approaches to the origins of thymic epithelial cells showed that, in birds, pharyngeal endoderm

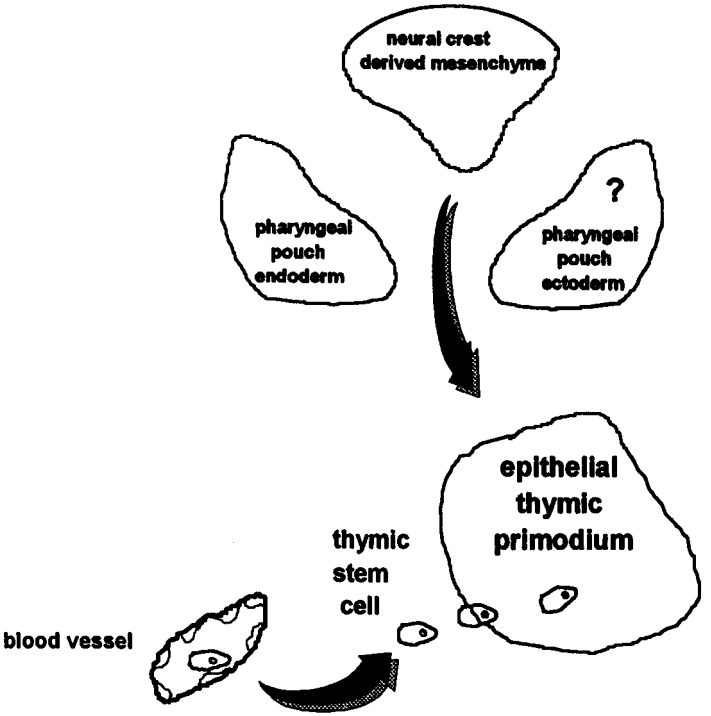

Figure 1 Developmental origins of the thymus.

dissected out without contamination by ectodermal cells could, in the presence of mesenchyme, produce full thymic histogenesis (7). Thus, the origin of thymic epithelial cells and the site of impact of the nude gene are still uncertain (Figure 1). In addition, the inductive effect of mesenchyme is also known to be necessary for thymic epithelial morphogenesis (8). The thymic primordium is surrounded by mesenchyme cells of neural crest origin (9), and experimental lesions of embryonic neural crest have been reported to result in defective thymic development (10). Finally, targeted mutation of the *Hox-A3* (*Hox-1.5*) gene results in an athymic embryo, among other craniofacial abnormalities (11). Whether this effect is mediated through epithelium and/or mesenchyme is unclear.

The embryonic thymic primordium is soon permeated by invading blood vessels and associated mesenchyme—here of mesodermal origin. Initially, there is no division into thymic cortex and medulla, but as lymphopoiesis proceeds these distinctive areas appear with their own types of epithelial cells (Figure 2).

Other important stromal cell types, such as dendritic cells and macrophages, are derived from precursors that enter the thymus from blood vessels. Macrophages are distributed throughout the cortex and medulla where they are involved in phagocytosis of apoptotic cells (12). Dendritic cells are predominantly located in medullary regions, but they reach into cortico-medullary areas and have access to immature thymocytes (13, 14). Within the architecture of the thymus, immature $CD4^-8^-$ thymocytes are situated in outer cortical regions, whereas $CD4^+8^+$ thymocytes are distributed throughout the remainder of the cortex. $CD4^+$ and $CD8^+$ T cells are most evident in the medulla, and from here they migrate to peripheral lymphoid tissues.

Origin and Development of Thymus Lymphocytes

The old arguments about the origins of thymus lymphocytes—Are they derived from thymic epithelial cells, mesenchymal cells surrounding the thymus, or from stem cells arriving via the blood stream?—were resolved with the introduction of cell transfer experiments combined with cell marker systems (15–17). The results clearly showed that stem cells reach the thymus through the blood, migrating through vessel walls to enter the thymic stroma. However, these studies left unanswered questions concerning the nature of thymic stem cells: Are they pluripotential or already restricted to T cell development when they enter the thymus, and what is the nature of the chemotactic factor involved in attracting stem cells to the thymus?

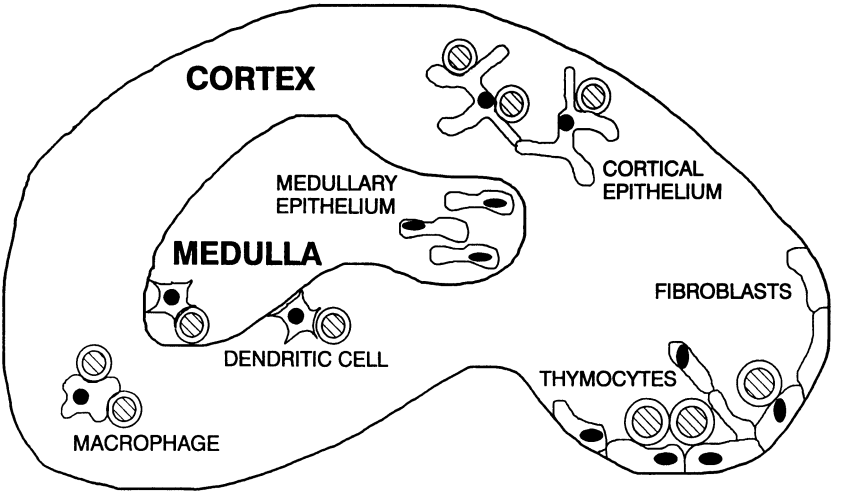

Figure 2 Stromal components of the thymic microenvironment.

Once in the thymus, stem cells proliferate and undergo gene rearrangement to produce $\gamma\delta$ and $\alpha\beta$ T cells. For $\alpha\beta$ cells, a number of control points regulating further development have now been defined. In particular, expression of the pre-T cell receptor (pre-TCR) is required for progression from the $CD4^-8^-$ to the $CD4^+8^+$ stage, while positive selection, mediated by the TCR$\alpha\beta$, is needed for the development of $CD4^+8^+$ thymocytes into single positive $CD4^+$ or $CD8^+$ T cells (18, 19). Choice of lineage between CD4 and CD8 also occurs at positive selection. The relative importance of stochastic and instructional mechanisms in this process has been extensively reviewed in recent times and is not considered further in this review except to note that the mechanism involved in the generation of $CD4^+$ and $CD8^+$ cells may differ with the downregulation of CD8 involving a stochastic mechanism, whereas the downregulation of CD4 is an instructional event (20, 21).

Thymocyte–Stromal Cell Interactions

While it has long been known that the thymic microenvironment is uniquely efficient in supporting the development of T cells, the nature of the support provided by the thymus has not been clear. However, progress has now been made in defining the role of individual thymic stromal components at different stages of T cell development (22). Similarly, the cellular interactions mediating repertoire selection, particularly in the case of positive selection, are becoming more clearly defined (23, 24). In parallel, gene knockout studies are also providing new insights into the intracellular signaling events regulating differentiation and selection (25, 26). Intriguingly, evidence is also accumulating to show that thymocyte/stromal cell interaction is a two-way process in which the development and maintenance of stromal cell function is dependent upon the influence of developing thymocytes (27).

In the following sections some of these issues are dealt with in more detail, with particular emphasis on the role of thymic stromal cells in T cell maturation and on the signaling processes regulating T cell development.

COLONIZATION AND CELL KINETICS IN THE THYMUS

Mechanisms of Precursor Recruitment

Pluripotential cells have recently been shown to first arise simultaneously in the yolk sac and para-aortic splanchopleura of 8.5-day mouse embryos (28, 29). Both sites contain stem cells that can colonize the thymic primordium (28–30), but whether they contain T lineage–committed precursors as well as pluripotent stem cells is unknown. Thus the maturational status of T cell precursors entering the thymus remains a major unresolved issue in T cell development. Precursors with the potential to give rise to $\alpha\beta$ T cells, $\gamma\delta$ T cells, NK cells, B cells,

macrophages, and dendritic cells have all been detected in the thymus (31–33). However, in the absence of clonal assays, the extent to which this reflects the entry of separate lineage committed/restricted precursors, of pluripotential precursors, or of both is uncertain.

That pluripotential hemopoietic precursors can directly enter the thymus is strongly suggested by the ability of 8–9-day yolk sac cells to colonize "empty" fetal thymus lobes in vitro (30, 34). Clear evidence has also been obtained recently to show that pre-thymic, T lineage–committed as well as multipotential precursors are present in the fetal blood of day-15.5 embryos (35). Whether or not both precursor types normally enter the thymus, and if so, whether they contribute differentially to the successive waves of precursor entry seen during development (36) or differentially to the formation of $\gamma\delta$ and $\alpha\beta$ T cells remains open to speculation. However, there is some recent evidence that $\gamma\delta$ and $\alpha\beta$ T cells can share a late common intra-thymic precursor (37).

Colonization of the thymus during development is initiated before vascularization of the thymic rudiment, so that blood-borne precursors have first to leave the pharyngeal vessels and then to traverse the surrounding mesenchyme to reach the thymus. Currently, the mechanisms directing this migration are poorly understood. A role for β-6 integrins in mediating the adhesion of prothymocytes to endothelial cells has been demonstrated (38), suggesting that these molecules may play a role in the initial emigration of precursors from the peri-thymic vessels. Similarly, a role for CD44 in the homing of i.v.-injected adult thymic precursors back to the thymus has been shown by antibody blocking studies (39), although the site of action of this effect and the CD44 ligand(s) involved are unknown.

Functional studies, using transfilter migration assays to assess the ability of thymic rudiments to attract precursors from various sources in vitro, have provided evidence that chemoattractant factors are produced by the thymic stroma (40, 41). Such factors are thought to form a gradient attracting precursors through the surrounding mesenchyme, and they could act in "soluble" or matrix-bound form as demonstrated for proteoglycan binding chemoattractant molecules in other systems (42). Presentation of thymic chemoattractant in bound form on the luminal surface of adjacent vessels could also be important in signaling the exit point for circulating precursors.

Previous suggestions that β_2-microglobulin (β_2m) might play a role in recruitment to the thymus seem unlikely in view of normal thymus colonization in $\beta_2\mathrm{m}^{-/-}$ mice (43). However, with the demonstration that chemokines (notably MCP-1) can act as chemoattractants for mature T cells as well as monocytes (44), it will be interesting to examine the response of thymus colonizing precursors to known chemokines and to investigate individual thymic

stromal components for chemokine production. Variation in factor output in response to changes in the size of the intrathymic precursor pool could be important in controlling the recruitment of new precursors and will be open to direct examination once the factor(s) are identified.

Cellular Proliferation in the Thymus

While continued precursor recruitment from the hemopoietic tissues is necessary to replenish the intrathymic precursor pool, intrathymic proliferation plays an important role in providing a sufficiently large number of TCR$\alpha\beta^+$ cells on which repertoire selection can operate. Previous studies have established that proliferation is mostly associated with the CD4$^-$8$^-$ and the early (i.e. TCR$^-$) CD4$^+$8$^+$ populations (reviewed in 45). Thus most cortical CD4$^+$8$^+$ TCR$^+$ thymocytes are small nondividing cells. These cells have a finite lifespan of 3–4 days but, contrary to previous assumptions, are not exclusively dead-end cells and can undergo positive selection and differentiation into single positive CD4$^+$ or CD8$^+$ cells (46, 47). Moreover, continued TCRα chain gene rearrangement has been demonstrated in this population, maximizing the opportunities for positive selection (48). The latter, although driven by TCR ligation, does not directly result in the proliferation and clonal expansion of the selected cells (49). However, there is recent evidence that single positive cells may undergo some limited division before leaving the medulla, where they can reside for up to 14 days (45). The significance of this proliferation and whether it is antigen driven or antigen independent is currently unknown.

Recently, more detailed information on proliferation within the CD4$^-$8$^-$ compartment has been obtained using CD44 and CD25 to define subpopulations and BrdU incorporation to detect cell division (50). The information shows that cells that are largely nondividing following entry into the thymus undergo two phases of proliferation separated by a period of quiescence at the CD44$^-$25$^+$ stage. Interestingly, TCRβ-chain–deficient mutants also undergo arrest at the CD44$^-$25$^+$ stage (see below), consistent with the notion that signals involving the pre-TCR complex at this stage are involved in triggering the second wave of proliferation.

Both stem cell factor (SCF) and IL-7, which are produced by thymic stromal cells (51, 52), appear to play a role during these early proliferative phases as shown by markedly reduced thymocyte numbers in IL-7$^{-/-}$ mice (53) and by antibody blocking of the SCF ligand c-kit (54–56). The relative involvement of these factors in the first and second proliferative waves remains to be clarified, although the pattern of c-kit expression suggests that SCF is primarily involved in the first wave (54, 56).

Finally, an inverse correlation between proliferation and TCR gene rearrangement has been observed, with initial expression of the fully rearranged β-chain

gene occurring during the phase of proliferative quiesence at the $CD44^{-}25^{+}$ stage, while expression of fully rearranged β- and α-chain genes is associated with the later nondividing $CD4^{+}8^{+}$ population (reviewed in 57). This seems to reflect transcriptional control of RAG gene expression, which also occurs in two waves correlating with these two periods of proliferative quiescence, with a decrease in expression in the intervening proliferative phase (58). In addition, a further mechanism limiting gene rearrangement mainly to periods of quiescence is suggested by the observation that RAG-2 protein accumulates in the G0/G1 phase of the cell cycle but is reduced during S, G2, and M, possibly as a result of degradation consequent upon phosphorylation by cyclin-dependent kinases (reviewed in 57).

THYMOCYTE-STROMAL INTERACTIONS REGULATING T CELL DEVELOPMENT

Interactions between the thymic microenvironment and immature thymocytes are known to play a critical role during T cell development. By providing a combination of cell-cell contact and soluble factors, thymic stromal cells provide signals to developing thymocytes, which can ultimately lead to proliferation, induction of differentiation, or cell death by apoptosis. Thus, characterization of the thymic stroma and elucidation of the specific functions of individual stromal cell types is fundamentally important in understanding the regulatory mechanisms that mediate T cell development.

Phenotypic Heterogeneity of Thymic Stromal Cells

Phenotypic heterogeneity and characterization of the thymic stroma have been reviewed extensively in recent years (27, 59). However, the degree to which this phenotypic heterogeneity is a reflection of functional specialization of thymic stromal cells is still unclear. Thymic stromal cells within subcapsular, cortical, and medullary regions consist of a complex mixture of cell types, including epithelial cells, mesenchyme cells, macrophages, and dendritic cells (60). Several studies have shown that the epithelial component of the thymic stroma plays a major role in T cell development, most notably by providing MHC antigens that regulate the selection of the TCR repertoire (61). However, the developmental origins of different thymic epithelial subsets is unclear. A recent study by Ropke et al has identified a thymic epithelial cell that shares phenotypic properties of both mature cortical and medullary epithelium (62). Such cells appear in the 14–16-day embryonic thymus but are absent from neonatal thymus, suggesting that cortical and medullary subsets of thymic epithelium may have a common precursor. Thymic epithelial cells are also heterogeneous with regard to MHC antigen expression. Interestingly, expression of MHC class-I

Table 1 phenotypic and functional characterization of thymic stromal cell subsets

Reagent	Species	Thymic reactivity	Function	References
HS9	Mouse	50-kDa molecule on cortical epithelium	Blocks maturation at $CD4^-8^-$ stage in FTOC	(63)
MTS23	Mouse	120-kDa molecule on medullary epithelium	No effect in FTOC. Costimulatory molecule?	(64)
ERTR4	Mouse	Unknown determinant on cortical epithelium	Inhibits thymocyte-epithelial cell binding	(65)
NLDC145	Mouse	205-KDa endocytic receptor on cortical epithelium and dendritic cells	Antigen processing	(66)
J4-81	Human	100-kDa glycoprotein on epithelium cells	Ligand for CD6	(67)
WGA	Chicken	Cortical and medullary epithelium	Unknown	(68)
Con A	Chicken	Cortical stroma	Unknown	(68)
Galectin	Human	Epithelial cells	Binding to CD45 on thymocytes?	(69)
PT10B7	Rat	Cortical and a subset of medullary epithelium	Unknown	(70)
B13D11	Rat	Medullary epithelium	Unknown	(70)

products on thymic epithelial cells is lower than that of MHC class-II products, which is at a level comparable to that seen on bone marrow–derived dendritic cells (23). However, not all thymic epithelial cells express MHC class-II antigens. For example, while cortical epithelial cells are MHC class II^+, medullary epithelial cells that react with the antibody A2B5 are not (23). Although the functional significance of this heterogeneity is uncertain, it is interesting to note that MHC class-II^+, but not MHC class II^- thymic epithelial cells are essential for the maturation of T cell precursors (22).

Several studies have used a variety of reagents including antibodies and lectins to investigate phenotypic and functional heterogeneity within stromal cell subsets (Table 1). For example, Takeuchi et al (63) have raised a monoclonal antibody HS9 that recognizes a determinant on the surface of thymic cortical epithelial cells. Moreover, addition of HS9 to fetal thymic organ cultures, recolonized with fetal liver cells as a source of T cell precursors, results in a blockade of thymocyte maturation at the $CD4^-8^-$ stage, suggesting that interactions between epithelial cells and thymocytes involving the determinant

recognized by HS9, are important for maturation to the $CD4^+8^+$ stage. However, because of this early blockade of maturation, the potential effect of HS9 on positive selection could not be examined. However, reaggregate cultures, in which thymic stromal cells are reassociated with individual thymocyte subsets, can be used to investigate the mechanisms regulating particular stages of thymopoiesis independently of other developmental events. The use of reaggregate cultures in the study of T cell maturation is described in detail in later sections.

Modulation of the Thymic Microenvironment by Developing T Cells

A fascinating aspect of thymus biology to emerge in recent years is the requirement for contact with thymocytes for the generation of functional stromal microenvironments. Thus, interactions between thymocytes and stromal cells are essential for the development of both T cell precursors and the thymic stroma. The first experiments to describe this phenomenon involved treatment of mice with cyclosporin A (CsA) (71), following which depletion of medullary epithelial cells was observed. At the time, the reasons for this unexpected effect on thymic stromal cells was unclear. However, when similar findings were reported in SCID mice, this absence of medullary development was conclusively shown to be caused by a defect in the lymphoid compartment of the thymus, since the introduction of normal bone marrow into SCID mice resulted in the regeneration of medullary epithelial cells (72). Thus, normal development of the thymic medulla is dependent upon the presence of thymocytes. More recently, absence of medullary areas has been noted in TCRα-deficient mice (73). Because these animals show normal T cell development with the exception of a lack of mature single positive $\alpha\beta$ T cells due to a defect in positive selection (74), it appears that interactions involving $CD4^+$ and $CD8^+$ TCR$\alpha\beta^+$ thymocytes regulate development of the thymic medulla. Although the nature of the interactions regulating medullary organization is not clear, a recent study by Shores et al suggests that a fully assembled TCR/CD3 complex may be required (75) to promote normal development of medullary epithelium.

Until recently, a role for similar mechanisms in regulating cortical epithelium development had not been defined. However, a study by Hollander and colleagues suggests that normal development of thymic cortical epithelial cells is dependent upon contact with thymocytes (76). Thus in mice expressing a CD3ϵ transgene, which results in a blockade in thymocyte maturation at a very early prothymocyte stage, normal cortex development is not observed. Moreover, the requirement for this interaction between the thymic cortex and prothymocytes is developmentally restricted, in that wild-type prothymocytes

rescue normal development of cortical stromal cells in fetal but not adult mice. Thus, it appears that thymocytes play a role in the development of both cortical and medullary thymic areas.

Extracellular Matrix and Integrin-Mediated Interactions with the Thymic Stroma

It is well known that extracellular matrix proteins play a major role in cell migration, differentiation, and proliferation (77, 78). Thus, a key feature of the thymic microenvironment may be the production of extracellular matrix (ECM) components. Several studies have documented the localization of a number of ECM molecules in the thymus, including type I and type IV collagen, laminin, fibronectin, vimentin, and merosin (77, 79). Although a fine network of ECM exists in subcapsular, cortical, and medullary areas, expression of particular matrix molecules appears to be prominent in particular sites in the thymus (77).

Whether compartmentalization of the ECM is important for T cell development is not clear. However, production of ECM in the thymus is mirrored by the expression of receptors for these molecules on developing thymocytes, suggesting that interactions between T cell precursors and ECM molecules may play a role in T cell maturation. Interestingly, several studies have shown that ECM receptor expression is highest on immature $CD4^-8^-$ precursors and gradually decreases during maturation. For example, Wadsworth and colleagues (80) showed that expression of the $\alpha_6\beta_4$ integrin, which binds laminin, reached maximal levels on $CD4^-8^-25^+$ precursors. In a further study (81), Wadsworth et al showed that expression of VLA integrins—which consist of a common β_1-chain and one of six α-chains—is developmentally regulated, and that changes in the glycosylation state of these receptors may determine their precise role in T cell development. Recently, the $\alpha^E\beta_7$ integrin, which binds to E-cadherin, has been shown to play a role in the adhesion of intraepithelial lymphocytes to epithelial cells in the intestine (82). Interestingly, this integrin receptor is also expressed on the majority of 14-day embryonic $CD4^-8^-$ T cell precursors (our unpublished observations). Whether or not this molecule also plays a role in the adherence of immature thymocytes to thymic epithelial cells is unknown. However, a more defined role has been established for the LFA-1 integrin. Fine & Kruisbeek (83) have shown that interactions between the LFA-1 integrin on thymocytes and ICAM-1 on either thymocytes or thymic stromal cells play an important role in the maturation of $CD4^-8^-$ thymocytes to the $CD4^+8^+$ stage.

Functional Roles of ECM in Early Thymocyte Development

Although the localization of ECM molecules and their receptors in the thymus is well characterized, evidence for their functional role in T cell maturation is less abundant. Our own studies have shown that mesenchyme cells, a major

producer of ECM in the thymus (84), are an essential requirement during the initial stages of T cell precursor maturation (22). In an attempt to elucidate the role of mesenchyme cells during thymopoiesis, we have used reaggregate organ cultures to map the precise developmental stage at which the requirement for these cells occurs (our unpublished observations). The results suggest that the first T cell precursor population to be independent of mesenchyme support are $CD4^-8^-25^+44^-$ cells, while the immediate precursors of this population, $CD4^-8^-25^+44^+$ cells, are still dependent upon interactions with mesenchyme. Thus, downregulation of the CD44 molecule marks the end of the mesenchyme-dependent stages of T cell development. Whether or not CD44 has a functional role during early intrathymic development is still unclear, although anti-CD44 antibodies have been shown to block the stromal cell–dependent stages of B cell development (85).

The molecular nature of the requirement for mesenchyme cells is still unclear but could involve the interaction of ligands on lymphoid cells with various mesenchymal ECM products such as hyaluronan, collagen, and fibronectin (86–88). In addition, Roberts et al (89) have shown that 3T3 fibroblasts—which can substitute for embryonic mesenchyme during T cell development—retain the ability to promote maturation and proliferation of hemopoietic stem cells, even when metabolically inactivated. These data are consistent with the idea that mesenchyme cells may influence the maturation of lymphocyte precursors by providing ECM products, which may in turn be important in the presentation of soluble growth factors, as is seen during the development of myeloid cells (90).

Thymic Stromal Cells and Selection of the TCR Repertoire

Immature $CD4^+8^+$ thymocytes bearing low levels of the TCR$\alpha\beta$ are subjected to stringent positive and negative selection events such that only cells bearing receptors with the potential to recognize foreign peptides in the context of self-MHC molecules are allowed to mature into single positive $CD4^+$ or $CD8^+$ T cells. Interactions involving the TCR on thymocytes and MHC molecules on the thymic stroma are known to play a critical role in thymic selection (61). A major factor in determining the outcome of TCR ligation during T cell development appears to be the avidity of the interaction between the TCR and self-peptide MHC complexes. Thus, low avidity interactions lead to positive selection and further differentiation, while high avidity interactions lead to negative selection and death by apoptosis (91, 92). However, it is also clear that TCR-mediated interactions alone are not sufficient to promote either positive (24) or negative selection (93), so factors other than the avidity of TCR-MHC interactions must contribute to T cell selection. Thymic stromal cells are known to play a crucial role during T cell selection, and it is likely that particular stromal components provide appropriate accessory signals that may determine whether a particular

$CD4^+8^+$ thymocyte undergoes positive or negative selection. The following sections summarize recent findings regarding the role of thymic stromal cells in T cell selection.

STROMAL CELL REQUIREMENTS FOR POSITIVE SELECTION The first experiments to investigate positive selection of thymocytes involved the production of MHC-mismatched bone-marrow chimeras, which suggested that a non-bone marrow–derived, radioresistant thymic stromal component was responsible for triggering the maturation of immature thymocytes into mature single positive T cells (reviewed in 19). While these findings were subsequently contested (reviewed in 94), it was generally agreed that thymic epithelial cells mediated positive selection of thymocytes. However, this conclusion was recently questioned again by a number of studies suggesting that a variety of cell types could mediate positive selection (95). These experiments, involving either the construction of bone marrow chimeras using β_2m-deficient mice (96), or the intrathymic injection of MHC class I–bearing fibroblasts into β_2m–deficient mice (97), indicated that thymic epithelial cells were not alone in their ability to promote positive selection of thymocytes to the $CD8^+$ lineage. However, when the developmental requirements of $CD4^+$ thymocytes were investigated, markedly different results were obtained. Thus, while Hugo et al demonstrated maturation of $CD4^+$ cells in MHC class II–deficient mice injected intrathymically with MHC class-II^+ fibroblasts (98), Glimcher et al were unable to rescue positive selection of $CD4^+$ cells by introducing MHC class-II^+ bone marrow–derived cells into MHC class-II deficient mice (99).

These experiments suggest that, at least under some conditions, various cell types can provide MHC ligands for positive selection. However, because they are based on intrathymic injection, they do not rule out the possibility that endogenous stromal components may cooperate in providing signals that may be essential during positive selection. To rule out this possibility, we used reaggregate cultures where the ability of individual thymic and nonthymic stromal components to support positive selection could be tested in the complete absence of additional stromal support (24). Of the cells tested for the ability to support maturation of $CD4^+8^+$ thymocytes, including MHC class-II^+ thymic dendritic cells, salivary epithelial cells, and fibroblasts, our study indicated that the ability to support maturation to either the $CD4^+$ or $CD8^+$ stage was a property unique to MHC class-II^+ thymic cortical epithelial cells (24). Moreover, thymic epithelial cells were still able to support maturation, albeit less efficiently, after chemical fixation, suggesting that positive selection is mediated by cell surface ligands rather than soluble factors. Recently, using bispecific antibodies, Muller & Kyewski have provided further evidence in support of a unique role for thymic epithelial cells in positive selection; they show that targeting of $CD4^+8^+$

thymocytes specifically to CDR1$^+$ thymic epithelial cells can rescue development of functionally mature CD4$^+$ cells in MHC class II–deficient mice (100).

Recently, we have extended our studies on epithelial cell/thymocyte interactions in positive selection by examining the duration of interactions required for the process to go to completion (our unpublished observations). By separating CD4$^+$8$^+$ thymocytes into CD69$^-$ and CD69$^+$ subsets, we have been able to compare the developmental requirements of thymocytes at a preselection stage (CD69$^-$ cells) with that of cells that have received initial signals for positive selection (CD69$^+$ cells). In agreement with our earlier study (24), we find that preselection CD4$^+$8$^+$ thymocytes are dependent upon contact with thymic epithelial cells for maturation to the CD4$^+$ or CD8$^+$ stage. Interestingly, this specific requirement for thymic epithelial cells is still evident at the CD4$^+$8$^+$69$^+$ stage, even though these cells have received initial signals for positive selection. Thus, these data suggest that during positive selection, thymic epithelial cells are required to provide sustained rather than transient interactions with CD4$^+$8$^+$ thymocytes. Similar conclusions have also been demonstrated in two other studies using an in vivo approach (101, 102). However, whether this requirement for sustained interaction involves TCR-MHC or other undefined interactions is not clear. We are currently investigating this issue by assessing the developmental potential of purified CD4$^+$8$^+$69$^+$ thymocytes in the presence of thymic stromal cells prepared from MHC class I– or class II–deficient mice.

Thus, the majority of experimental evidence suggests that thymic epithelial cells are the most efficient thymic stromal cell type capable of mediating positive selection of CD4$^+$8$^+$ thymocytes. However, very little is known about the accessory interactions that mediate maturation of CD4$^+$8$^+$ thymocytes. Intriguingly, although positive selection of thymocytes and activation of mature T cells are phenotypically similar in a number of ways (103), we have shown that these processes are not mediated by the same accessory interactions, since positive selection does not require interactions between CD28 and B7 (104). Elucidation of the accessory molecules that play a role during positive selection is a major goal of future research.

STROMAL CELL REQUIREMENTS FOR NEGATIVE SELECTION Study of the cellular and molecular requirements for negative selection of CD4$^+$8$^+$ thymocytes has benefited from the establishment of a number of in vitro culture systems (reviewed in 105). However, conflicting results have been reported regarding the identity of the thymic stromal cells that mediate negative selection, depending upon the experimental system used. For example, using suspension cultures, a number of studies have indicated that thymic epithelial cells can induce thymocyte apoptosis as efficiently as bone marrow–derived dendritic cells (106–109). However, as thymocytes in suspension have been shown to respond

differently to a given stimulus when compared to thymocytes in situ (110), the relevance of findings obtained in such cultures to events in vivo remains unclear.

Using reaggregate organ cultures, we showed that effective deletion of SEB reactive Vβ8$^+$ CD4$^+$8$^+$ thymocytes was achieved by bone marrow–derived dendritic cells (23). Under similar conditions, MHC class-II$^+$ thymic epithelial cells were found to be less efficient at mediating negative selection, suggesting a specialization of function for epithelial cells and dendritic cells in mediating thymocyte apoptosis. However, prolonged exposure to SEB presented by thymic epithelial cells was found to effect maturation of Vβ8$^+$ thymocytes. Moreover, a recent study by Spain & Berg (111) using TCR transgenic mice in which MHC class-II expression in the thymus is restricted to epithelial cells has suggested that cortical epithelial cells may indeed play a role in the induction of self-tolerance. However, whether the mechanism of tolerance in this case was induction of apoptosis or anergy is unclear. Moreover, these experiments do not exclude the possibility that, while thymic epithelial cells may provide TCR ligation for negative selection, bone marrow–derived APC cooperate in providing accessory signals in a three-way interaction similar to that discussed for positive selection.

Again using reaggregate organ cultures, Merkenschlager and colleagues (112) have investigated the number of bone marrow–derived antigen-presenting cells required to induce maximal deletion of TCR transgenic thymocytes. Such studies showed that in contrast to positive selection, where a linear relationship exists between the number of selecting stromal cells and the number of thymocytes induced to mature, far fewer APCs per thymocyte were required to induce maximal deletion, suggesting that these cells form multiple contacts with developing thymocytes during development and are highly efficient mediators of negative selection.

As in positive selection, the molecular nature of the interactions between immature thymocytes and thymic stromal cells that lead to negative selection are unknown. However, as dendritic cells are also efficient mediators in the activation of mature T cells, it is possible that interactions important in activation are also involved in thymocyte selection. One such interaction is between the costimulatory molecule B7 on dendritic cells, and CD28, which is expressed on both mature T cells and thymocytes. However, Jones et al (113) have shown that CTLA4Ig, a high-affinity soluble ligand for B7, which effectively blocks mature T cell activation, does not block negative selection of thymocytes either in vivo or in thymic organ cultures. Finally, using purified thymic stromal cell preparations and comparing the cell type–specific expression of different mammary tumor virus antigens (Mtv) with the pattern of TCRVβ deletion in thymic organ culture, we have shown a correlation between differential TCRVβ

deletion and differential expression of Mtv antigens by thymic dendritic cells even when Mtvs were expressed by other stromal cells (114). This supports the notion that the influence Mtvs and possible other proteins have on shaping the T cell repertoire depends not only on their presence but also on their tissue-specific expressions and presentation on appropriate APCs.

Although it is well known that only a small minority of immature thymocytes reach full maturity, an aspect of T cell development that has received little attention is the mechanism by which apoptotic thymocytes are cleared from the thymic microenvironment. Using the TUNEL technique to identify DNA strand breaks in thymocytes undergoing apoptosis, Surh & Sprent (12) showed that a complex network of macrophages, present in both cortical and medullary regions, is highly effective at the phagocytosis of thymocytes triggered into apoptosis. Interestingly, it appears that the majority of cells in the thymus are not triggered into apoptosis as a consequence of negative selection but are a product of a death-by-neglect process, in which thymocytes unable to undergo positive or negative selection are eliminated. Although the molecules involved in phagocytosis of apoptotic thymocytes are unknown, it is interesting to note that cortical macrophages express a variety of adhesion molecules such as VCAM-1 that could regulate thymocyte/macrophage interactions (115).

SIGNALING EVENTS CONTROLLING THE DEVELOPMENT OF TCR$\alpha\beta$-POSITIVE CELLS

Signaling events involving different forms of the TCR complex appear to be important during at least two key stages in T cell development. First, expression of a pre-TCR consisting of the TCRβ chain paired with the pre-TCRα chain (also known as gp33) and elements of the CD3 complex is necessary for the progression from $CD4^-8^-$ to $CD4^+8^+$ stage. Second, interactions involving the definitive TCR$\alpha\beta$ are necessary for positive selection and differentiation of $CD4^+8^+$ cells to mature single positive thymocytes.

Signaling in $CD4^-8^-$ Cells

T cell precursors, which enter the thymus with the majority of their TCR genes still in germline form, are induced to rearrange their TCR genes during the $CD4^-8^-$ phase of development (116). Although this involves the expression of RAG-1 and RAG-2 proteins (as discussed earlier), the signals initiating RAG expression and TCR gene rearrangement are still unclear. A role for IL-7 has been proposed in this respect (117); however, IL-7 also promotes the expansion and survival of $CD4^-8^-$ thymocytes (18, 118). Consequently, the possibility that IL-7 maintains cells in which TCR gene rearrangement has already been induced cannot be excluded.

A range of studies now clearly shows that prevention of successful TCRβ chain rearrangement, either due to the naturally occurring severe combined immunodeficiency (SCID) mutation or the inactivation of V(D)J recombination activation genes (RAG-1 or RAG-2) by gene knock-out technology, results in strongly reduced thymic cellularity and a block in T cell development at the $CD4^-8^-$ stage (119, 120). Development to the $CD4^+8^+$ stage can be restored either by introducing a fully rearranged TCRβ transgene (121, 122) or by treatment with anti-CD3 (123, 124), implying that signaling through a putative TCRβ/CD3-containing complex expressed on the surface of $CD4^-8^-$ thymocytes mediates early T cell development. Whether this signaling simply requires assembly of this complex or interaction with an as-yet-unknown ligand is still to be defined. Whichever is the case, pre-TCR-mediated signals appear to be vital for the clonal expansion and further differentiation of thymocytes that have productively rearranged their TCRβ chain genes (125, 126). Moreover, additional events associated with normal differentiation from $CD4^-8^-$ to $CD4^+8^+$ such as the arrest of rearrangement at the TCRβ locus (i.e. allelic exclusion), the initiation of TCRα chain rearrangement, and CD4 and CD8 coreceptor expression have now, at least in part, been linked to signaling through this putative TCRβ/CD3-containing "pre-TCR" complex (125–127).

A TCRβ-transfected immature T cell line from SCID mice in which the TCRβ transgene is expressed on the cell surface in an 80-kD disulphide-linked complex, has been used to determine the nature of this "pre-TCR" more closely (128). In these cells the TCRβ is paired to a glycosylated chain of 33 kD which, because of its properties, has been named the pre-TCRα chain. This pre-TCRα is a type I transmembrane protein, the extracellular region of which comprises about 130 residues, with similarity to the constant (C) domain of immunoglobulin supergene family proteins. Sequence analysis indicates that it is well suited for pairing with TCRβ. The cytoplasmic region of about 31 amino acids contains a possible SH3-domain binding region and two potential phosphorylation sites for protein kinase C, and the region may directly be involved in signal transduction by the pre-TCR (129). Expression of pre-TCRα does not require rearrangement and is strong in $CD4^-8^-$ thymocytes from normal mice (129). Moreover, a very recent study shows that knocking-out the pre-TCRα gene by homologous recombination severely hampers TCR$\alpha\beta$ development (130). This confirms that while the pre-TCR is not required for the generation of $CD4^-8^-25^+$ thymocytes, few cells progress beyond this stage. Thus the pre-TCR is necessary for the efficient generation of $CD4^+8^+$ precursors.

The pre-TCR is associated with members of the CD3 family of proteins, and the resulting complex clearly has signaling capacity (124, 127). Although the means by which the pre-TCR complex exerts its action remain to be fully

elucidated, the lymphocyte-specific tyrosine kinase $p56^{lck}$ is likely to be a part of pre-TCR signaling pathway. Much of the evidence for this has come from studies with mice that are either $p56^{lck}$-deficient (131), carry a dominant negative $p56^{lck}$ mutation (132), or overexpress $p56^{lck}$ (133). For example, expression of an active $p56^{lck}$ gene in rearrangement-deficient (134) or normal mice (133) has the same effect as introducing a TCRβ transgene. Thymocytes in mice expressing the $p56^{lck}$-dominant negative mutation fail to develop beyond the $CD4^-8^-$ stage (132). Introduction of a TCRβ transgene in the latter animals leads to the development of small numbers of $CD4^+8^+$ cells, implying that the expansion of $CD4^+8^+$ thymocytes rather then their differentiation from $CD4^-8^-$ precursors is controlled by a $p56^{lck}$-sensitive pathway (134).

A role for Vav in early signaling events has been implicated by three recent studies in which Vav expression in cells of the lymphoid lineages is compromised (135–137). Under normal circumstances cross-linking of TCR results in the rapid tyrosine phosphorylation of Vav, a putative $p21^{ras}$ exchange protein. Tyrosine phosphorylation of Vav appears to stimulate, in vitro at least, its guanine nucleotide exchange activity which, in turn, contributes to the regulation of $p21^{ras}$ activity (see Figure 3 and Ref. 138). Although thymocyte differentiation to the $CD4^+8^+$ stage occurs in the absence of Vav, the number of cells in the thymus is severely reduced, indicating a role for Vav in thymocyte expansion (135–137).

Not much is known yet about the other members of this pre-TCR signaling pathway, although calcineurin may be involved since CsA affects development of $CD4^+8^+$ from their $CD4^-8^-$ precursors (our unpublished observations). It has, however, been shown that the $p21^{ras}$/Raf/MEK/ERK pathway, a common downstream-modulator of $p56^{lck}$ and Vav activation (Figure 3, and Ref. 139) is not involved (see below).

Selection and Differentiation of $CD4^+8^+$ Cells

It has been clear for some time that both positive and negative selection encroach upon the same population of $CD4^+8^+$ thymocytes. Much work has gone into explaining this apparent paradox, and several excellent reviews covering this area have been published recently (19, 140). Yet, whatever the underlying mechanism, it is clear that signaling through the TCR can have dramatically different consequences for $CD4^+8^+$ thymocytes, indicating the possible involvement of biochemically distinct signaling pathways.

Much of the information regarding TCR-mediated signaling processes has come from studies with mature T cells (Figure 3). TCR-mediated signaling events are complex and involve, among others, tyrosine phosphorylation mediated by $p56^{lck}$, $p59^{Fyn}$T, Syk, and ZAP-70, dephosphorylation mediated by CD45, and activation of the $p21^{ras}$ pathway (138, 141, 142). With the

development of gene-manipulated animals in which the activity of some of these molecules is either knocked-out or enhanced and the availability of more specific inhibitors of signal transduction molecules, the involvement of the various signaling pathways in T cell development is becoming more apparent.

Such studies show clearly that not all signaling molecules important in mature T cell activation similarly play a role in T cell development. The PTK $p59^{fyn}$ T, for example, is associated with the TCR and is vital for mature T cell activation but is not needed for full T cell maturation. Limiting $p59^{fyn}$ T activity has no discernible effect on selection (143). Moreover, PKC activity which is vital for mature T cell activation is not essential for either positive or negative selection, as shown by the lack of effect of the specific PKC inhibitor Ro 31.8425 on these processes (144). The development of $CD4^+8^+$ cells from $CD4^-8^-$ precursors is also unaffected by Ro 31.8425 (our unpublished observations).

As discussed above, the PTK $p56^{lck}$ plays an important role in early T cell development; however, its role in selection of $CD4^+8^+$ cells has not been fully elucidated. $p56^{lck}$ associates with the cytoplasmic tail of both CD4 and CD8 co-receptor molecules (145), but this association appears not to be an

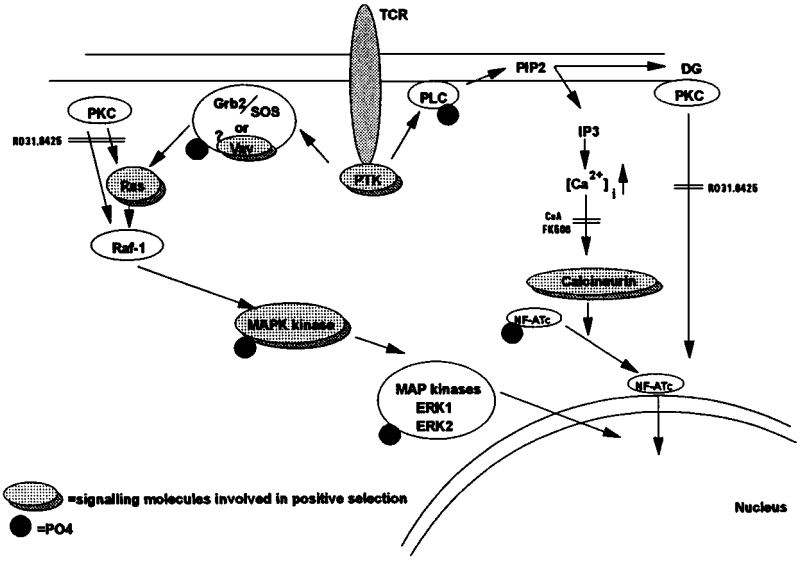

Figure 3 TCR signal transduction pathways. PTK-mediated phosphorylation results in: (i) the activation of PIP^2 signaling and (ii) the activation of the $p21^{ras}$/Raf-1/ERK-2 signaling cascade. $p56^{lck}$, $p21^{ras}$, MAPK kinase, calcineurin, Vav, and CD45 phosphatase (the latter not shown in this diagram) are all potentially involved in mediating signals for positive selection.

absolute prerequisite for selection since the reconstruction of $CD4^{-/-}$ mice with a tailess form of CD4 unable to bind $p56^{lck}$ restores $CD4^+$ SP cell development (146). Similarly CD8 does not need the lck-binding motif on its cytoplasmic tail to promote $CD8^+$ SP cell development (147). However, overexpression of $p56^{lck}$ in TCR transgenic animals, in which the effects of $p56^{lck}$ on early T cell development are circumvented by the introduction of the fully rearranged transgenic TCR, reduces not only the level of TCR expression in $CD4^+8^+$ thymocytes but also the efficiency of positive selection. In contrast, despite these relatively low levels of TCR, negative selection occurs efficiently in these animals (148), indicating that when overexpressed the $p56^{lck}$ interferes with positive but not negative selection of $CD4^+8^+$ thymocytes.

Information regarding the involvement of ZAP-70 in T cell development has come from studies on those humans who fail to express ZAP-70 due to a mutation in the gene (149–151). Thymuses of patients contain both $CD4^+8^+$ cells, indicating that progression from $CD4^-8^-$ to $CD4^+8^+$ proceeds apparently as normal, and nonfunctional $CD4^+8^-$ SP thymocytes. Mature, single positive $CD8^+4^-$ SP cells are absent. ZAP-70, therefore, is indispensable for the development of SP $CD8^+$ cells as well as the signal transduction and function of SP $CD4^+$ cells.

CD45 is a protein tyrosine phosphatase that is exclusively expressed on nucleated cells of hematopoietic origin and is required for TCR-mediated signal transduction in mature T cells (152). Knocking-out the exon 6 domain of CD45 results in strongly reduced expression of CD45. This coincides with a reduction in positive selection, while superantigen-induced negative selection is unaffected (153), indicating that CD45 activity may be required differentially for positive and negative selection.

$p21^{ras}$ is a low molecular weight G protein that is known to participate in signaling from a wide variety of tyrosine kinases and has been strongly implicated in signal transduction from the TCR in mature T cells (Figure 3) (138). Suppressing $p21^{ras}$ activity in T lineage cells through the introduction of a dominant negative form of $p21^{ras}$ under the control of the lck proximal promoter strongly reduces positive selection (25). In contrast, negative selection proceeds normally, indicating that the $p21^{ras}$ contributes to the regulation of positive but not negative selection of $CD4^+8^+$ thymocytes. Not surprisingly the MAPK kinase MEK-1, a downstream effector of $p21^{ras}$ (Figure 3), shows a similar involvement in positive selection (26). Development of $CD4^+8^+$ cells from their $CD4^-8^-$ precursors is not affected by the reduction in $p21^{ras}$ or MEK-1 activity (25, 26).

On the other hand, the role of the putative $p21^{ras}$ exchange protein Vav in selection is far from clear. Thymocyte expansion is clearly reduced in the

absence of Vav (see above). However, while Tarakhovsky et al (135) and Zhang et al (136) report normal selection in Vav-deficient mice, Fischer et al (137) report a block in the later stage of positive selection. A potential role for Vav in negative selection has not yet been investigated.

Differences between signaling requirements for positive and negative selection have also been observed using CsA and FK506 in defined systems of T cell development (144, 154). CsA and FK506 are immunosuppressive agents that inhibit mature T cell activation by inhibiting the activity of calcineurin, a calcium-, calmodulin-dependent phosphatase. Inhibition of calcineurin either in vitro (144) or in vivo (154) blocks positive selection but leaves negative selection unaffected.

Clearly, evidence is emerging in support of the hypothesis that positive and negative selection operate through biochemically distinct signaling pathways. Positive selection involves, among others, the activation of PTK, the $p21^{ras}$-dependent signaling pathway and calcineurin (Figure 3). In contrast, signaling pathways involved in negative selection are far from clear. There is some evidence that phosphatidylionositol metabolism is involved in antigen-induced deletion (155). However, we have shown that, within the normal thymic microenvironment, TCR ligation by ConA induces a level of hydrolysis comparable to that induced by anti-CD3 under similar circumstances. Yet, in contrast to anti-CD3, ConA does not induce apoptosis (our unpublished observations). Therefore, PIP_2 hydrolysis may be essential but is not sufficient to TCR-mediated cell death in the thymus.

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ONE STEP AHEAD OF THE GAME: Viral Immunomodulatory Molecules

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ABSTRACT

For decades cell biologists have relied on viruses to facilitate the study of complex cellular function. More recently, the tragedy of the AIDS epidemic has focused considerable human and financial resources on both virology and immunology, resulting in the generation of new information relating these disciplines. As the miracle of the mammalian immune system unfolds in the laboratory, the elegance of the mechanisms used by co-evolving viruses to circumvent detection and destruction by the host becomes inescapably obvious. Although many observations of virus-induced phenomena that likely contribute to the virus's escape of immune surveillance are still empirical, many other such phenomena have now been defined at the molecular level and confirmed in in vivo models. Immune modulators encoded within viral genomes include proteins that regulate antigen presentation, function as cytokines or cytokine antagonists, inhibit apoptosis, and interrupt the complement cascade. The identification of such gene products and the elucidation of their function have substantially strengthened our understanding of specific virus-host interactions and, unexpectedly, have contributed to the recognition of potent synergy between viruses, which can result in an unpredictable exacerbation of disease in co-infected individuals. Because many viral immune modulators clearly have host counterparts, viruses provide a valuable method for studying normal immune mechanisms. It is conceivable that an improved understanding of virus-encoded immunomodulators will enhance our ability to design reagents for use in therapeutic intervention in disease and in vaccine development.

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INTRODUCTION

The clinical syndromes resulting from virus infection have interested scientists for centuries. The identification of virus particles as entities separate from bacteria, detailed serological classification, in vitro culture systems, and the development of animal models have solved much of the mystery surrounding mammalian virus infections. However, one of the most dramatic accomplishments in the field of virology unquestionably has been at the level of biochemistry, with the adventitious advances in nucleic acid sequencing.

While scientists today argue the ethics of determining the nucleotide sequence of the human genome, virologists have already moved forward, shamelessly laying open entire viral genomes for public scrutiny. The ease and reproducibility of nucleic acid sequencing techniques has allowed, among other things, more precise taxonomic classification, insight into transcription, translation, and replication mechanisms at both the viral and cellular level, and critical insight into viral epidemiology and vaccine development through the expression of recombinant, immunogenic viral proteins.

Evidence supporting sophisticated interactions between viruses and the immune systems of their hosts has increased, and attention has turned sharply toward many undefined viral genes known as open reading frames (ORFs). Computer-assisted comparisons of viral genome sequences against cumulative databases have revealed significant homology between several viral ORFs and mammalian genes whose products participate in immune or inflammatory cascades. In the last few years, many of these viral homologues have been confirmed as functional modulators of immune responses in vitro. Where viruses themselves can be genetically manipulated and animal models are available, some immune modulating ORFs play a role in the generation of virus pathogenesis in vivo. The identification of viral immune modulators is not restricted, however, to recognition of homology between viral ORFs and host genes. In fact, the elucidation of the function of a viral protein with no recognizable homology to known proteins can lead to the identification of novel mammalian gene products.

The mechanisms that viruses use to perpetuate themselves within the host appear reflected in the varied immune modulators known to be encoded in viral genomes. Such modulation includes selective regulation of host antigen presentation, production of growth factors that function on host cells, and antagonism of immune function through the use of soluble versions of cytokine receptors. In many cases, these viral proteins function to assist the virus in avoiding host immune surveillance, while in other instances they clearly function to protect the host from the sometimes deleterious effects of the virus-induced inflammatory response.

This chapter attempts to provide an overview of some of the more recently identified virus-encoded immune modulators. The functional descriptions in this text are arranged loosely in an order that might parallel the temporal progression of the cell-mediated arm of the adaptive immune response (e.g. antigen presentation, CTL formation, cytokine production, and apoptosis). Obviously, virus infections elicit potent, innate immune responses as well, and examples of functional virus-encoded complement protein homologues have existed for years. For the purposes of this chapter, however, the modulation of innate responses is addressed primarily at the level of antagonism of cytokines and chemokines. In addition, there are many examples of virus-specific regulators of the humoral response, including functional homologues of Fc receptors and the use of virus-specific host mimicry, which are not discussed here. Thus, this chapter does not provide a truly comprehensive description of the impressive work in the field of virus-host interactions. [Several specialty reviews addressing this rapidly expanding discipline have been written recently and are highly recommended reading (1–8).] The final portion of this chapter touches upon the clinical considerations that may arise as a result of the production of potent viral immune mediators during active infection, particularly in the immunocompromised patient.

REGULATION OF ANTIGEN PRESENTATION

Interference with MHC Class I Function

Cellular immunity has long been recognized as critical in the host control of virus infections. The recognition and subsequent destruction of infected cells by natural killer (NK) cells and virus-specific cytolytic T lymphocytes (CTL) provide a first line of defense against most viruses. Virus-specific CTL can be detected within a few days after infection and typically peak prior to production of high-affinity virus-specific antibody. CTL not only assist in virus clearance through the elimination of infected cells, but they are also required to control persistent virus infections, reactivation of latent infections, and virus-associated tumor growth.

Most virus-specific CTL recognize infected cells through their T cell receptor (TCR)–CD8 cell surface complex, which binds to the major histocompatibility complex (MHC) class I molecules expressed on the surface of virtually all somatic cells. When a cell is infected, the MHC class I molecules display, or present, peptide antigens derived from degraded intracellular viral protein. CTL recognition of the foreign viral antigen in the context of the "self" MHC molecules leads to cellular activation, resulting in both lysis of the infected

cell and production of cytokines and other growth factors that allow for the expansion of the virus-specific CTL population.

A successful virus infection requires sufficient time for genome replication and production of infectious progeny in order to allow for systemic or cell-cell spread. CTL antigens often represent immediate early or early viral protein, i.e. protein made shortly after infection, prior to the onset of genome replication. Recognition of early protein-derived peptides might be advantageous because it facilitates the destruction of infected cells prior to production of progeny virions. However, examples of vigorous CTL responses against peptides derived from late viral antigens (i.e. viral proteins synthesized after onset of viral genome replication) have also been described and are also likely to be important in the control of virus spread within the host. Thus, it seems logical that a diminished CTL response might provide a selective advantage in the establishment of viral infection. Indeed, many viruses have evolved mechanisms for downregulation of class I MHC expression.

Lytic infection with many viruses, including herpesviruses, adenoviruses, and vaccinia virus (VV), can result in decreased surface MHC expression as a result of the overall shut off of host protein synthesis by the input virus. While this may represent an effective strategy for diminishing a CTL response, such cells die rapidly and therefore represent a limited resource to the infecting virus. Some viruses, however, have developed more subtle strategies for interfering with MHC expression.

Herpesviruses are considered among the most successful of viruses, with seroconversion rates of greater than 90% in some instances. Not surprisingly, herpesviruses are considered masters of host immune evasion, employing a variety of strategies that ultimately allow the establishment of latent infections and occasional reactivations that ensure efficient perpetuation of the virus within a population. Most herpesviruses exist in relative harmony with the host; however, during acute infection or reactivation, particularly in the immunocompromised individual, the virus-specific pathology is sufficiently severe to maintain the herpesviruses as a clinically relevant group.

Herpesviruses are grouped into three subfamilies, referred to as α-, β- and γ- herpesviruses. The α-herpesviruses include the prototypic herpes simplex virus (HSV) and varicella zoster virus (VZV), the causative agent of chicken pox and shingles. β-herpesviruses include murine cytomegalovirus (MCMV) and human cytomegalovirus (HCMV). HCMV infections are responsible for an array of clinical syndromes and are of particular concern in pregnant women and immunocompromised individuals such as transplant recipients and HIV-infected patients. The third group, the γ-herpesviruses, exhibit very strict host range, and latent infection of these viruses occurs only in lymphoid cells. This

subfamily includes Epstein-Barr virus (EBV), the causative agent of infectious mononucleosis, known also to be associated with some forms of Burkitt's lymphoma and nasopharyngeal carcinoma; a T-lymphotropic primate virus, herpesvirus saimiri (HVS); and the newly isolated herpesvirus associated with Kaposi's sarcoma (9). Both NK cells and CTL activity are necessary to maintain human herpesvirus infections in a subclinical form. Interference of normal T cell function can lead to virus reactivation (10–12). The modulation of MHC class I expression has been reported for HSV (13, 14), EBV (15–18), and CMV (7).

HSV provides an elegant example of interference with virus-specific CTL recognition through regulation of MHC class I/peptide complex expression. HSV-1 and HSV-2, like all herpesviruses, shut off MHC class I expression late in infection as a result of overall diminished host protein synthesis. Similarly, NK cells and CTL can be inactivated or killed as a result of cell-cell virus spread. However, more specific mechanisms are working in HSV: Researchers have found that anti-HSV-specific CTL isolated from infected patients are frequently $CD4^+$, MHC class II restricted, rather than the more common $CD8^+$, MHC class I-restricted type, and that HSV-infected fibroblasts are poorly recognized by HSV-specific $CD8^+$ CTL (19, 20). This lack of recognition can be reversed by the addition of virus-specific peptide (19). York et al (21) have shown that MHC class I proteins are retained in the endoplasmic reticulum (ER) of HSV-infected fibroblasts within 3 h post infection and, using HSV deletion mutants, that the protein product of the ICP47 gene is necessary for this retention. Furthermore, co-infection of human fibroblasts with recombinant adenoviruses expressing ICP47 and HCMV diminishes HCMV-specific $CD8^+$ CTL recognition and killing compared to cells co-infected with a control recombinant adenovirus and HCMV, thus indicating that the ICP47 protein is also sufficient for the phenotype.

ICP47 is a cytosolic protein, which, coupled with the obvious block in MHC class I transport and maturation, suggests that the mechanism of inhibition of class I expression may be occurring at the level of association of peptide with nascent class I molecules. The TAP (transporter associated with antigen presentation) genes are localized within the MHC and include two distinct loci that encode the TAP1 and TAP2 proteins (22–24). The TAP proteins function as a heterodimeric complex (25, 26) binding to cytosolic peptides and facilitating their transport across the membrane and into the lumen of the ER, where they associate with class I/β_2-microglobulin (β_2m) heterodimers. The correct maturation and transport of MHC class I molecules is dependent on their association with peptide, and experiments from two independent groups have indicated that ICP47 interferes with the function of the TAP1/TAP2 complex.

Immunoprecipitation (27, 28) and immunofluorescence (27) experiments indicate that ICP47 co-localizes with the TAP1/TAP2 complex intracellularly. Using separate approaches, the two groups have unequivocally shown that the association of ICP47 with the TAP1/TAP2 complex inhibits the transport and association of peptide with MHC class I, resulting in rapid degradation of the empty class I molecules. To date, this is the only example of this type of regulation of antigen presentation by a virus, and it also represents the only known regulator of TAP function other than intracellular ATP levels.

More information on mechanisms of class I regulation has come from studies in HCMV. Previous work has implicated an HCMV ORF termed UL18 that exhibits significant homology to MHC class I (29) as playing a role in interfering with normal MHC class I transport and function (30–34). However, the creation of a mutant HCMV lacking a functional UL18 gene has resulted in a virus that is unimpaired in its ability to shut down the synthesis of mature MHC class I heterodimers following infection, indicating that the product of the UL18 gene is not responsible for the virus-specific decrease in class I expression (35). Recent work by Kim et al (36) has demonstrated that two separate loci, which function independently of one another, are responsible for the diminished class I phenotype of HCMV-infected cells. One of these loci encodes the early gene product US11, a 32-kDa cytosolic glycoprotein. Stable expression of US11 in human foreskin fibroblasts has resulted in decreased MHC class I expression as determined by western blot analysis. The exact mechanism remains to be determined; however, the cellular localization of US11 suggests that this gene may also function to inhibit the surface transport of MHC class I molecules, resulting in their rapid degradation. The function of the second locus involved in HCMV-mediated class I regulation has yet to be determined.

Other well-studied examples of MHC class I regulation by viruses are those utilized by various serotypes of adenoviruses. In humans, these viruses lytically infect epithelial cells and are responsible for various febrile respiratory syndromes. Adenoviruses are not believed to be oncogenic in humans; however, tumorigenicity of some serotypes in rodents has been clearly established (37).

An early observation by Kvist et al (38) indicated that a viral protein termed gp19 binds to MHC class I molecules in adenovirus type 2 transformed rat cells. The gp19 protein is encoded within the E3 region of the adenovirus genome, a term referring to a transcriptional unit containing approximately nine genes that are transcribed early after infection. The gp19 protein is a transmembrane protein (39), and the amino acid residues involved in binding to MHC class I have been mapped (40–42). This protein resides in the ER, presumably due to a retention signal in the cytoplasmic domain (43–46). The binding of gp 19

to nascent MHC class I heavy chains results in impaired intracellular transport as indicated by differential sensitivity to endoglycosidase H digestion and flow cytometric analysis of cell-surface expression (47–49). Interestingly, the gp19 protein appears to exhibit some allelic specificity of class I binding in both the murine and human systems (49–51). Whether this specificity plays a role in susceptibility to infection or manifestation of disease is unknown; however, such speculation is highly provocative.

In vitro studies from a number of different laboratories have shown that expression of the gp19 protein results in decreased sensitivity to virus-specific CTL killing in vitro (49, 50, 52, 53), suggesting that the downregulation of MHC class I by gp19 is one method by which adenoviruses might escape or delay the cell-mediated immune response. Consistent with this, one would predict that deletion of the gp19 gene from the adenovirus genome would result in the creation of a mutant virus that does not downregulate class I early after infection. This mutant virus should therefore be attenuated, likely being cleared more rapidly than wild-type virus due to enhanced CTL recognition. Interestingly, in vivo studies have indicated that this is not the case. Using an adenovirus-susceptible animal model, and human adenovirus type 5 mutant viruses defective for gp19 expression, Ginsberg et al (54) have reported an increased inflammatory response in animals infected with gp19-deleted viruses compared to animals infected with wild-type adenovirus, although there was no significant difference in mortality between the groups. The explanation for these results is not clear; however, due to the early onset of the inflammatory response in animals infected with the mutant virus, an altered cytokine response may have contributed to the enhanced pathogenicity (55).

In related studies, Grunhaus et al (56) expressed the adenovirus type 2 gp19 protein in recombinant VV and then used these viruses to infect either mice expressing H-2 haplotypes known to bind to the gp19 protein or mice expressing H-2 haplotypes that did not bind gp19. In these experiments, no detectable difference in virus replication, host lethality or CTL response was observed within the groups, compared to responses to control recombinant VV. The data from these two in vivo studies suggest some caution should be exercised in the interpretation of the function of gp19 based on in vitro results. However, it is also equally reasonable to assume that it may sometimes be difficult to interpret the analysis of a complex in vivo host immune response to viral infection in order to determine single gene function.

The E3 gp19 is not found in two adenovirus subgroups, A and F. Nonetheless, cells transformed with these viruses still downregulate MHC class I very early after infection. These viruses have developed an independent mechanism for regulation of class I that resides at the transcriptional level.

Tumors generated by adenovirus type 12 (Ad12), a highly oncogenic member of the A subgroup of adenoviruses, have been used to establish a direct correlation between tumorigenicity and decreased MHC class I expression (57–59). The Ad12 E1A region (an early transcriptional unit) contains a 13S mRNA required for the transcriptional downregulation of the MHC class I gene (60–63). Schouten et al (64) have recently shown that cells transformed with the Ad12 E1A region express reduced amounts of the p50 subunit of the transcription activating factor NFκB. This p50 subunit arises as a result of the proteolytic processing of a 105-kDa precursor, termed p105-NFκB. While p105-NFκB is cytoplasmic, the p50 cleavage product is found primarily in the nucleus, where it is known to bind to gene regulatory sequences, including a class I regulatory element that functions as an enhancer for MHC class I promoters (65, 66). The overexpression of a cDNA encoding a truncated, active version of the p105-NFκB that can substitute for wild-type p50 is able to reverse the Ad12 E1A-induced downregulation in transformed baby rat kidney cells. In addition, the adenovirus type 12 E1A region has been reported to decrease the transcription of the MHC-encoded peptide transporter genes TAP1 and TAP2 (67), although the mechanism of this regulation remains obscure.

The existence of MHC class I–associated negative regulatory nucleotide sequences that are able to bind viral proteins and turn off class I transcription has also been suggested. Deletion analysis of the mouse H-2K^b gene has been used to map regions required for downregulation of class I to sequences between -1.2 kb and -1.44 kb relative to the 5′ cap site. These sequences appeared to bind adenovirus encoded and/or induced proteins, although those proteins have not been specifically identified (68, 69).

Regulation of MHC Class II Expression

In contrast to the ubiquitous MHC class I, MHC class II molecules are more restricted in their expression, being found primarily on antigen presenting cells such as dendritic cells, macrophages, B cells, and (in the human) activated T cells. MHC class II molecules typically display exogenously acquired peptides that are recognized by the TCR-CD4 complex, thereby activating T helper cells.

Very little evidence is available that suggests specific regulation of MHC class II expression following virus infection. Many viruses may indirectly regulate MHC class I and II expression through the release of virally encoded cytokines or antagonism of cytokines by virally encoded soluble cytokine receptors (discussed later in this chapter). However, recent work suggests that mechanisms other than regulation of expression may be used to modulate MHC class II function.

Lewandowski et al (70), using two strains of HSV that differ in their pathogenicity, have examined MHC class II expression following infection. HSV-1 strain

F typically establishes a latent infection following intraocular inoculation (71–74). In contrast, inoculation with HSV-2 strain KOS results in a lethal infection. Staining of microglial sections using a mAb specific for the murine MHC class II I-E gene product (the equivalent of the human HLA-DR protein) indicated aberrant, nuclear localization of MHC class II in KOS-infected cells, whereas normal cytoplasmic class II staining was observed for MHC class II in HSV-1 strain F infected cells.

Based on these results, it was postulated that the KOS-induced aberrant expression of cell-surface MHC could result in diminished antigen presentation during infection. As a result, T helper–cell activation and subsequent cytokine production would also be affected in KOS-infected animals. This hypothesis was examined using RNAse protection assays to ascertain levels of cytokine mRNA in brain tissue from HSV-2 KOS-infected animals compared to HSV-1–infected animals. Lewandowski et al have determined that KOS-infected samples have significantly diminished levels of IL-6 and γ interferon (γIFN) transcripts compared to HSV-1–infected tissues, while both groups of animals have comparable levels of tumor necrosis factor (TNF) or IL-1 mRNAs. Although the cytokine mRNA detection was performed on whole brain tissue, these data have been interpreted to support the hypothesis that $CD4^+$ T cells in KOS-infected mice are less activated during infection as a result of diminished MHC class II expression (75).

Another viral protein known to interact directly with MHC class II is the product of the BZLF2 ORF of EBV. BZLF2 encodes a type II membrane glycoprotein that is expressed late during lytic infection. The BZLF2 ORF, expressed in a recombinant form as a soluble Fc fusion protein, binds to β chains of the MHC class II locus. Inhibition of binding of soluble BZLF2 using mAbs to different MHC class II molecules indicates preferential binding to HLA-DR compared to HLA-DP or HLA-DQ. Direct binding studies on deletion mutants of the HLA-DR β chain demonstrate that BZLF2 recognition requires the β chain domain that participates in the formation of the peptide binding pocket, and in vitro assays indicate that the affinity of binding of soluble BZLF2 to peripheral blood lymphocytes is sufficient to block MHC class II–directed antigen presentation. The BZLF2 protein can bind to the HLA-DR heterodimer intracellularly as well as on the cell surface, and it may function to retain MHC class II in the ER. However, separate experiments have shown that BZLF2 is transported efficiently to the cell surface when co-expressed with two additional EBV glycoproteins, and that BZLF2-specific mAbs that block the interaction of this protein with MHC class II prevent the infection of MHC class II positive B lymphocytes (76). Thus, during normal EBV infection, BZLF2 may play a dual role, modulating class II–directed antigen presentation and facilitating infection of class II positive cells.

In addition to the MHC regulation by viral proteins such as those described in this section, it should be remembered that regulation of MHC protein expression is also highly dependent upon cellular cytokines such as γIFN and TNF, which are often produced in response to infection. Ultimately, then, the regulation of MHC expression in the infected cell may depend on both virus-encoded and virus-induced factors that exist in a dynamic state.

REGULATION OF THE CYTOKINE NETWORK

Viral Cytokines

Cytokines are potent, soluble proteins that play key roles in the induction and maintenance of inflammation, immune response, differentiation, and embryonal development. Many of these effects are a result of cytokine-mediated gene regulation. The description of a soluble factor that inhibits the production of cytokines produced by Th1-like murine T cells has led to the isolation of a cDNA encoding what is now called IL-10 (77, 78). The deduced amino acid sequence of human IL-10 (hIL-10) exhibits 78% identity overall to the putative product of the BCRF1 gene of EBV, a 17–kDa protein that is produced and secreted during the lytic phase of EBV infection (79, 80). Not surprisingly, hIL-10 and BCRF1 have similar biological functions. Recombinant hIL-10 and BCRF1 both inhibit monocyte/macrophage activation and subsequent cytokine synthesis, and the synthesis of γIFN by T cells and NK cells, which interferes with γIFN-induced upregulation of MHC class II on antigen presenting cells. In addition, they both inhibit other γIFN-induced antiviral pathways such as nitric oxide production. In contrast to these inhibitory effects, both hIL-10 and BCRF1 are efficient stimulators of B cell proliferation in vitro (81). Thus, BCRF1 likely facilitates EBV replication through the activation of resting B cells and also assists EBV in escaping host immune surveillance by downregulating inflammatory cytokines.

Interestingly, while the biological effects of hIL-10 and BCRF1 are strikingly similar, they are not identical; they exhibit differential effects on various murine T and B cells (78, 82–84). Although it is possible that this merely reflects a difference in a diminished binding affinity of BCRF1 for the murine IL-10 receptor (mIL-10R) compared to that of hIL-10, additional data suggest that the explanation may be more complicated. Inhibition-of-binding studies indicate that BCRF1 exhibits a 100–1000-fold poorer affinity for the IL-10Rα chain compared to the cellular cytokine. However, neutralizing mAb against the hIL-10Rα (85) blocks the response of the hIL-10R transfectants to BCRF-1, demonstrating a requirement of the IL-10Rα chain for BCRF-1 responses (KW Moore, personal communication). Taken together, a likely interpretation

of these data is that the hIL-10R exists as a complex that consists of as yet unidentified proteins that can signal as a result of binding of BCRF-1.

Another example of a viral cytokine is found in HVS. Previous work has identified an mRNA derived from rodent T cells that was predicted to encode a 15–kD secreted protein. This mRNA contains nucleotide sequence motifs in its 3′ untranslated region similar to those found in several cytokine mRNAs. These two observations suggested that this T cell–derived mRNA might represent a novel cytokine. A comparison of the sequence of this mRNA (which is referred to by its discoverers as CTLA8) with others in the cumulative database revealed significant homology between CTLA8 and the thirteenth ORF of HVS (HVS-13) (86).

These observations have been recently extended by Yao et al (87, 87a). Immunoprecipitation experiments using mAbs against recombinant HVS-13 protein have confirmed the presence of HVS-13 in HVS-infected cell supernatants (87). Cytokine-like activity for HVS-13 has been demonstrated by the induction of NFκB on, and IL-6 production by, fibroblast cell lines (87, 87a), and also by the ability of HVS-13 to costimulate murine T lymphocytes (87, 87a). A comparison of the biological activities of HVS-13 on either murine or human cell types suggests that HVS-13 is more potent on cells of human origin (87, 87a). This may reflect the fact that since HVS is a virus whose tropism is restricted to primates, HVS-13 likely represents the viral homologue of a human CTLA8-like molecule. At present, the precise function of HVS-13 in relation to HVS infection is unknown; however, its T cell mitogenic activity may facilitate the infection of these cells by HVS.

Viral Growth Factors

A series of growth factors has been identified among members of the poxvirus family. Poxviruses are large, DNA-containing viruses, and although they are no longer considered clinically relevant human pathogens, the family includes one of the most destructive historical viral pathogens, smallpox (variola virus), as well as one of the most successful vaccine immunogens, VV. Computer-assisted sequence comparisons identified significant homology between a 19-kDa VV protein (vaccinia growth factor, or VGF) and members of the epidermal growth factor (EGF) family. EGF and related molecules bind to a membrane glycoprotein receptor, and this ligand-receptor interaction can initiate cellular signaling cascades that include tyrosine phosphorylation and activation of second messenger systems. The most common biological response is a proliferative one by the cell engaging the EGF-like ligand. Like EGF, VGF binds the EGF receptor and is directly mitogenic (88–90). Deletion of the VGF gene from the VV genome and analysis of the mutant virus in the permissive chorioallantoic membrane model suggest that VGF may function to stimulate hyperplasia at the site

of infection, facilitating cell-cell spread of the poxvirus. Deletion of the VGF homologue in rabbit poxviruses and subsequent infection of rabbits indicate that the VGF gene product contributes to the pathogenicity of these viruses (91, 92).

Another group of poxviruses contains ORFs whose potential protein products are related to vascular endothelial growth factor (VEGF) (93). VEGF mediates its activity through a specific receptor (94, 95) and plays an important role in angiogenesis. The poxviruses that carry these VEGF-related ORFs cause pustular dermal lesions that show vascular endothelial proliferation after virus infection of the natural hosts (96). Although the viral VEGF homologues are transcriptionally active, they have not yet been purified and analyzed directly for activity.

ANTAGONISM OF HOST CYTOKINES

Virus infection at the cellular level is seldom subtle, and the host response to these invading pathogens is typically robust. Most viruses induce cellular cytokines as a result of infection, particularly those cytokines of the IL-1, TNF, and IL-8 families. These cytokines are often referred to as exhibiting "antiviral" activity, which is perhaps not unexpected considering the pleiotropic nature of these immune regulators. What is surprising are the intricate, multilevel strategies employed by viruses to cope with host production of these potent cytokines.

Various soluble mammalian cytokine receptors have been identified in serum and urine. These receptors arise from alternative splicing, as in the case of the IL-4 receptor (R) (97); the IL-7R (98); and the GM-CSFR (99) or proteolytic cleavage from the cell surface, as for the IL-6R (100) and the TNFR (101). The functions of soluble cytokine receptors appear diverse. Many can bind to their respective ligands with high affinity, suggesting antagonism of activity by blocking the attachment of the cytokine to a signaling cell–surface receptor. Interestingly, binding of a cytokine to a soluble receptor can also work to extend the half-life of a cytokine in vivo, as was shown for the TNFR (102) and the IL-4R (103). The soluble IL-6R is unusual in that a complex of this receptor and IL-6 can act as an agonist of IL-6 activity by binding to gp130, the signal-transducing component of the IL-6R complex (104). Clearly, the function of the soluble receptor may vary with the cytokine, but in each case the modulation of cytokine activity occurs prior to the binding of the ligand to the cell. To date, the strongest argument for a physiological role for soluble mammalian cytokine receptors comes from the identification of specific virus-encoded soluble receptors for several different cytokines.

The α (leukocyte) and β (fibroblast) IFN molecules (collectively referred to as type I IFNs) are induced by virtually every type of viral infection, presumably

through the induction of double-stranded RNAs (dsRNA). The type I IFNs mediate their antiviral activities through the induction of cellular proteins that in turn participate in RNA degradation, the inhibition of translation, and MHC antigen regulation (reviewed in 105). Two type I IFN receptors (IFNR) have been molecularly cloned (106–108), found to contain fibronectin-like repeats, and are considered members of the class II cytokine receptor family. Although both α and β IFN appear to bind to the same receptor (106, 108, 109), additional data indicating a functional dissociation of the IFN ligands (107, 110) suggest that another α/β receptor or associated chain may exist. The recent identification of a novel, soluble α/β IFNR in poxviruses (111, 112) lends strong support to the notion of an additional mammalian IFNR.

Supernatants from VV-infected, but not mock-infected, cells exhibit heterologous antiviral activity in that they can inhibit rhabdovirus infection of HeLa cells. Using a panel of deletion mutants of VV, the gene responsible for this phenotype has been mapped to a portion of the viral genome that contains two ORFs (111), both of which encode proteins whose predicted secondary structure classifies them as members of the immunoglobulin superfamily (113). One of these, B18R, is expressed at the surface of VV-infected cells (114) and in infected cell supernatants. The B18R protein was shown to bind α, β (111, 112) and ω (112) IFN from a broad species range (111). Although homology between B18R and the α subunits of the human type I IFNR have been reported (112), computer-assisted analysis of the B18R sequence strongly suggests an evolutionary relationship to the IL-1R or IL-6R (113, 115, 116). Thus, B18R represents an intriguing dilemma for structure-function experts. Does B18R represent an IL-1R-like molecule whose ligand-binding characteristics have been altered as a result of the more rapid evolution of the virus compared to the host? Or does it represent an unappreciated structural similarity between the IL-1R and cytokine receptor-like families (112)? The identification of a mammalian homologue for B18R and an analysis of its function will likely help solve these interesting questions.

Poxviruses also encode soluble receptors for type II IFN, or γIFN, a cytokine produced by NK and T cells. Besides possessing autocrine functions, γIFN activates macrophages, upregulating their MHC class II expression, and regulates B-lymphocyte differentiation (117). Like the type I IFNs, γIFN also induces the cellular proteins that ultimately lead to the inhibition of translation and the RNA degradation associated with "antiviral" activity.

The first poxvirus γIFNR was discovered when N-terminal sequencing of the most abundant protein in myxoma virus-infected cell supernatants allowed the identification of a viral ORF with limited, but significant, homology to the type II IFNR (118). Since then, homologues of the myxoma virus–encoded IFNR

have now been identified in a variety of poxviruses, including several strains of VV (119), smallpox (120, 121), and cowpox (122). These receptors appear to bind γIFN in a fairly species-restricted manner (123). The poxvirus γIFNRs exhibit very low overall homology to the murine or human type II IFNR, and whether these proteins represent viral homologues of the cloned mammalian receptor or a separate unidentified receptor is not clear.

Soluble, poxviral receptors have also been identified for TNFα and β (124) and for IL-1β (115, 116). These receptors appear to bind with high affinity and exhibit species preference if not specificity (115, 116, 125). The viral TNFR is most closely related to the cloned p75 TNFR, and like this protein, the viral TNFR binds efficiently to both TNFα and TNFβ (124). In contrast, the viral IL-1R is most closely related to the cloned type II IL-1R, but unlike that receptor, the viral IL-1R exhibits a clear preference for binding to IL-1β compared to IL-1α (115, 116).

Mutant viruses in which selective gene inactivation of the viral TNFR (myxoma virus) (126) or of the IL-1R (VV) (116) were generated and tested for altered pathogenicity in animal models. These in vivo studies are particularly interesting because they appear to illustrate two contrasting roles for virus-encoded soluble cytokine receptors. Infection of rabbits with the TNFR$^-$ mutant myxoma virus results in dramatic attenuation of disease compared to wild-type infection (126). This suggests that the viral TNFR functions to antagonize the "antiviral" immune properties of TNF. In contrast to these results, the intranasal infection of mice using VV with a selectively inactivated IL-1βR gene results in an increased morbidity, but not mortality, of infected animals (116). These latter experiments suggest that the viral IL-1βR may function to protect the host from the deleterious effects of excessive production of IL-1.

Virus-encoded, membrane-bound chemokine receptors have been identified also. Chemokines are a family of soluble proteins that regulate numerous inflammatory responses. The chemokines are classified into two groups based on their structure: the C-C chemokines which include MIP-1α, MIP-1β, MCP-1, and RANTES, and the C-X-C chemokines, which include IL-8, GRO, and NAP-2. HCMV contains an ORF termed US28 that exhibits 33% overall amino acid homology compared to the mammalian IL-8Rs. Expression of this US28 viral receptor in mammalian cells indicates that it binds members of the C-C family of chemokines and that it transduces a signal, suggesting that its function may not be antagonistic (127, 128). A similar ORF has been identified in the γ-herpesvirus, HVS. In contrast to the HCMV chemokine receptor, the HVS receptor binds members of the C-X-C family of chemokines, but not the C-C family (129).

Unfortunately, no readily available animal models exist for either HCMV or HVS. Thus, the need for these functional chemokine receptors during infection is unknown. There is one report that the addition of exogenous IL-8 can enhance HCMV replication in fibroblastic cells in vitro (130); however, clearer understanding may require the use of single gene deletions and some form of in vivo model.

The importance of the IFNs, TNF, and IL-1 in the control of viral infection is evidenced by the additional, independent strategies employed by some viruses to modulate the effects of these cytokines. Poxviruses are known to contain two other genes whose protein products regulate the cellular processes induced as a result of IFN binding to its cellular receptor. The first strategy is represented by the VV E3L protein, which functions to inhibit IFN-induced shutoff of protein translation. The target of the E3L protein is dsRNA, and binding of E3L to this RNA successfully competes the binding of the dsRNA-dependent cellular kinase DAI (131–133). In order for the DAI kinase to phosphorylate and inactivate the elF2α translation initiation factor, it must first bind to dsRNA, an interaction inhibited by the VV E3L protein. Similar anti-IFN activity has been documented for the reovirus $\sigma 3$ protein (134, 135).

The poxvirus K3L functions differently to inhibit DAI activity. This protein contains significant amino acid homology with the elF2α protein and can function as a competitive substrate to DAI, which helps overcome the IFN-induced translational block (136–139).

Studies with the adenoviruses have revealed three viral gene products that function intracellularly to prevent TNF-mediated lysis of infected cells (reviewed in 140). Deletion analysis has identified the adenovirus genes responsible for this protection from TNF, but their exact function is still undetermined. One of these genes, the E1B 19K protein, is discussed more fully in the following section on regulation of apoptosis.

IL-1β has also been targeted at the intracellular level by poxviruses. The product of the crmA gene in cowpox virus plays a role in the development of hemorrhagic pocks on virus-infected chorioallantoic membranes (141, 142). The crmA protein belongs to the serpin, or serine protease inhibitor, family of proteins (143). One of two identified substrates for crmA is the IL-1β converting enzyme (ICE) (144–146), a protease responsible for the conversion of the inactive pro-form of IL-1β to its active secreted form (147, 148). ICE is now known to be a multifunctional protease; however, its importance in the processing of IL-1β and, surprisingly, also in the production of IL-1α, is evidenced by the phenotype of mice in which the ICE gene has been disrupted (149). These mice are defective for mature IL-1β production, have diminished IL-1α compared to littermate controls, and are resistant to the induction of

endotoxic shock, a condition believed to result in large part from excessive IL-1 production. Thus, expression of the crmA protein likely functions during infection to inhibit the activity of ICE and, subsequently, to diminish production of mature IL-1β. Recent evidence indicates that as a result of targeting the ICE protease, the crmA gene plays an additional and quite separate role in immune evasion (discussed further in the next section).

REGULATION OF APOPTOSIS

Apoptosis, or programmed cell death, is a process by which a cell dies in response to certain internal or external stimuli. Morphologically, cells undergoing apoptosis exhibit alterations in their plasma membrane and nuclear structure, including discrete endonucleolytic cleavage of their chromosomal DNA (reviewed in 150–153). This process is essential both in development of the organism and in maintaining a balance among differentiated or activated cells. CTL recognition of virus-infected cells results in a rapid, apoptotic death of the target cells, providing an obviously important mechanism for the elimination of virus-infected cells. In many cases, however, cells undergo apoptosis as a direct result of the establishment of an active infection. The internal signals that trigger apoptosis following infection are not well understood; however, much has been learned recently about the viral genes aimed at the inhibition of this premature cell death.

The DNA fragmentation during apoptosis (154) that is now well documented was observed several years ago during infection with mutant adenoviruses that contained a deletion of the E1B 19K gene, but not in cells infected with wild-type adenovirus (155–158) (The E1B 19K protein is distinct from the E1A 19K protein described previously in this chapter's section on regulation of antigen presentation, and the two proteins are related only at the level of an unfortunate nomenclature). The E1B 19K protein is expressed relatively early after infection and is one of the three genes involved in the establishment of resistance of adenovirus-infected cells to TNF-induced cytolysis (140, 159). Expression of the E1B 19K protein also protects cells from apoptotic cell death induced as a result of unrelated adenovirus proteins (160) and Fas activation (161).

This inhibition of apoptosis is similar to that seen when bcl-2, a cellular proto-oncogene, is expressed. The bcl-2 gene was originally isolated at a chromosomal breakpoint found in a follicular B cell lymphoma (162–164). It functions to protect cells from a variety of apoptosis-inducing stimuli in vitro, and the creation of transgenic mice expressing bcl-2 in thymocytes has indicated that bcl-2 functions similarly in vivo (165, 166). Surprisingly, these transgenic mice delete autoreactive T cells normally, suggesting that genes other than bcl-2 are important in the regulation of apoptosis. Experiments have since led to the

isolation of bcl-2 related molecules that are either similar to bcl-2 in function (167) or act in opposition to bcl-2 (167, 168). At least some members of the bcl-2 family are believed to form dimers, and the ratio of heterodimerization to homodimerization may determine the cellular survival following delivery of apoptotic stimuli (168). Experiments in which the bcl-2 gene is inserted into adenoviruses deleted for the E1B 19K protein have resulted in suppression of infection-induced apoptosis, indicating that bcl-2 can substitute for E1B 19K protein during adenovirus infection (169, 170). In an attempt to delineate the mechanism of both the bcl-2 and the E1B 19K proteins, a yeast two-hybrid system has identified cellular proteins that specifically bind these gene products (170). cDNA clones have been identified that encode three distinct proteins designated Nip 1, 2, and 3, and they bind specifically to both bcl-2 and E1B 19K. Deletional analysis of the bcl-2 and E1B 19K genes indicate that the Nip proteins bind to discrete regions of homology shared by bcl-2 and 19K. The exact function of the Nip proteins remains to be elucidated.

Again, using a yeast two-hybrid system, Farrow et al (171) have isolated a cellular gene called bak (bcl-2 homologous antagonist/killer), which shares 28% amino acid identity with bcl-2. The bak protein binds to the E1B 19K protein and to a previously isolated bcl-2 homologue, but not to bcl-2 itself (167). The activity of the bak protein opposes that of bcl-2, and when overexpressed, bak accelerates apoptosis (171–173). Thus, the adenovirus E1B 19K gene may bind to this apoptotic accelerator and render it nonfunctional. Some pieces of the puzzle are still missing, however, as bak is expressed constitutively in numerous cells and has not been demonstrated to be induced upon infection.

Apoptosis plays an important role in B cell development, limiting the life span of most B lymphocytes (174–176). The expression of bcl-2 has been implicated in the selection of memory B cells (177). EBV is a B-lymphotropic virus, and expression of the EBV latent proteins in Burkitt's lymphoma cells appears to enhance cellular survival in the presence of apoptotic stimuli (176). This enhanced survival occurs as a result of bcl-2 induction (178). This bcl-2 induction protects latently infected B cells but cannot inhibit apoptosis of naive B lymphocytes as a result of primary EBV infection. This responsibility seems to fall instead to an early expressed EBV ORF referred to as BHRF-1.

BHRF-1 shares approximately 25% identity with bcl-2 (179) and exhibits strong homology to other bcl-2 family members (180). Expression of the BHRF-1 gene in Burkitt's lymphoma cells (181) or in Chinese hamster ovary cells (182) protects them from numerous apoptotic stimuli, including growth factor depletion, cisplatin or mitomycin C treatment. Deletion of BHRF-1 from the EBV genome does not impair its ability to replicate in or transform B cells in vitro (183, 184), implying that the BHRF-1 inhibition of apoptosis is most

important during lytic infection, keeping the cell intact long enough to permit the production of the progeny virions.

The ability of EBV to upregulate bcl-2 in addition to encoding its own bcl-2 homologue underscores the sensitivity of B lymphocytes to destruction via apoptosis. EBV does not exclusively infect B lymphocytes, however; the virus lytically infects epithelial cells of the oropharynx, which under certain circumstances may also become transformed. This is believed to be what happens in EBV-associated nasopharyngeal carcinoma. Another EBV-associated, epithelial-derived tumor is oral hairy leukoplakia (OHL), a benign lesion of the mucosa of the tongue. OHL lesions are sites of chronic EBV replication and do not appear to express the EBV genes associated with latency (185, 186). The BHRF-1 gene is abundantly expressed in OHL (187), and BHRF-1-mediated inhibition of apoptosis may interfere with normal epithelial differentiation resulting in dysplasia (188). Transfection of the BHRF-1 gene into a human squamous cell line results in delayed commitment of these cells to terminal differentiation as well as in resistance to apoptotic stimuli. Thus, like its bcl-2 cousin, BHRF-1 may sometimes function as an oncogene.

Earlier in this chapter, the poxviral crmA gene product was described as a member of the serpin family that could inhibit the maturation of IL-1β as a result of the inactivation of the ICE protease. ICE exhibits homology to the ced-3 protein (189). The ced-3, -4 and -9 genes, described in the nematode *Caenorhabditis elegans*, are required for proper development of this organism. ced-3 and ced-4 are required for apoptotic processes, while ced-9 (which is a member of the bcl-2 family) acts as an antagonist of apoptosis. The homology between ced-3 and ICE has suggested that in addition to its activity on pro-IL-1β, this mammalian protease may function in apoptosis (reviewed in 190). Overexpression of the ICE gene or the ced-3 gene in Rat-1 fibroblasts induces apoptosis in these cells, a process that is reversed by co-expression of either bcl-2 or the poxviral crmA gene (191). In other experiments, microinjection of the crmA gene into neuronal cells prevents their apoptotic cell death induced by nerve growth factor depletion (192). More recently, crmA was also shown to inhibit the activity of the CPP32β protein (144), a mammalian homologue of ced-3 (144, 193), which may be critical in the induction of mammalian apoptotic processes. Taken together, these data suggest that the poxviral crmA protein represents a single protein whose acquisition facilitates virus infection by helping evade immune surveillance at diverse levels.

CLINICAL CONSIDERATIONS

Several virus proteins have now been identified that regulate the mammalian immune response. Some of these proteins have biochemical characteristics

that suggest a therapeutic application. For example, the preferred affinity of the poxvirus-encoded soluble IL-1R for IL-1β compared to IL-1α might allow for a selective antagonism of an IL-1β-mediated portion of an IL-1 response. Also, because the soluble form of the EBV-encoded BZLF-2 blocks MHC class II–directed antigen presentation in vitro, it may be able to function as a general immunosuppressant if administered systemically. Furthermore, the nonidentical binding characteristics, but similar biological properties, of the viral cytokines compared to their cellular counterparts may result in their being less toxic than cellular cytokines.

This chapter describes experiments in which specific immune-modulating genes were inactivated in viruses and the pathogenicity of the mutant virus compared to that of wild-type virus. The measurable differences in pathogenicity noted in diverse virus mutants have profound implications for vaccine development as they suggest that by putting in, or taking out, select viral immune regulators, one may intelligently design virus vaccines that exhibit enhanced safety and efficacy. This concept is supported by an analysis of data regarding postvaccinial complications that resulted from the use of different strains of VV during the global eradication of smallpox. The ORFs that encode the soluble cytokine receptors found in VV are not conserved in the five strains used most frequently as immunogens. Vaccine recipients of VV strains that contain the soluble IL-1βR have fewer cases of postvaccinial encephalitis than those strains in which the IL-1βR ORF is disrupted. Because postvaccinial neurological complications are believed to be caused primarily by a host inflammatory response to viral antigen, and because IL-1 is a potent inducer of the inflammatory cascade, the vaccine strains containing a functional IL-1βR may be able to modulate a deleterious host IL-1 response (194).

Another consideration is the likelihood of direct clinical consequences arising as a result of the production of abundant, highly efficient, virus-encoded immune modulators by the infected patient. Although no direct data supports a role for virus-immune modulators in the exacerbation of disease in humans, information obtained from animal models strongly suggests that it likely occurs and that it may be particularly relevant in the immunocompromised host, where concurrent infection with multiple pathogens is common. For example, the avian models for co-infection of herpesviruses and retroviruses have been studies for several years using Marek's Disease Virus, a herpesvirus responsible for T cell lymphomas in chickens, and Avian Leukosis Virus, a retrovirus associated with a variety of neoplasms. Data obtained from this and related models clearly indicate that the production of viral protein by one pathogen can have profound effects on the transcription, replication, and tissue tropism of an unrelated virus infecting the same host (195 and references therein). The

avian co-infection model closely parallels the reactivation of herpesviruses in the AIDS patient, which represents a naturally occurring, active co-infection of two unrelated virus types (herpesviruses and retroviruses). During acute outbreaks, the infected individuals carry unusually high virus burdens, often with both virus types replicating in the same cell. Building on the findings from the avian system (195), investigators have now shown definitive examples of synergy between herpesviruses such as HSV, HCMV, or HHV-6 and HIV. Interactions include activation of retroviral LTR sequences by promiscuous herpesviruses transactivators, herpesvirus-mediated induction of the transcription factor NFκB (196–201), and in the case of HHV-6, specific upregulation of CD4 transcription, even in T cells which do not normally express CD4 (202). Thus, it is not unreasonable to postulate that virus-encoded MHC regulators, cytokines, cytokine antagonists, or inhibitors of apoptosis produced by one virus are likely to influence the outcome of disease caused by a co-infecting pathogen.

Synergistic interactions between different viruses may also be important where immunosuppression has been therapeutically induced. In these instances, the synergy may occur between different virus types within a given virus family. For example, the average incidence of reactivation of HCMV in renal allograft recipients is 85%, and in bone marrow recipients, the rate is approximately 60%. The pathology associated with HCMV reactivation in these populations includes fever, liver dysfunction, vasculitis, and lymphocytosis, making the pathogen a significant problem in transplant settings (203). Similarly, HHV-6 has a seroprevalence rate of over 85% and is increasingly recognized as a cause of hepatitis, encephalitis, pneumonia (204–206), and bone marrow suppression (207) in allograft recipients. Thus the probability is high that both of these herpesviruses will be reactivated simultaneously in the immunocompromised patient, resulting in high levels of virus replication, possibly in the same cell. Although these viruses are related, they are not identical, and each virus both carries and induces a unique set of immunomodulating proteins. This type of concurrent infection may introduce another variable worth considering when trying to predict clinical outcomes.

CONCLUDING REMARKS

The identification of virus-encoded proteins that interact with the host immune system is a rapidly expanding area of viral immunology. The elucidation of the function of these virus molecules will assist in the understanding of viral pathogenesis, provide tools with which to study and manipulate the normal host immune response, allow the design of safer virus vaccines and virus-based drug delivery systems, and permit us to prepare for the future when new "emerging" viruses will inevitably again test our ability to cope with the unexpected.

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GENETIC ANALYSIS OF TYROSINE KINASE FUNCTION IN B CELL DEVELOPMENT

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ABSTRACT

B lymphopoiesis is regulated by multiple signals from stromal cell contact, soluble cytokines, antigen, and T helper cells. In vitro and biochemical experiments have implicated tyrosine kinases as key components of many of these signaling pathways. Genetic analysis of the role of these tyrosine kinases has been facilitated by recent advances in transgenic and gene targeting technology as well as by the identification of the genetic basis of several human and murine immune deficiencies. This review discusses the effect of gain and loss of function mutations of selected tyrosine kinases and their regulators and substrates on B cell development and function.

INTRODUCTION

Proper function of the immune system is dependent on the differentiation of pluripotential hematopoietic stem cells into B cells capable of recognizing a diverse repertoire of antigens. This complex, tightly regulated process occurs in response to numerous signals from the bone marrow microenvironment, soluble cytokines, antigen, and T helper cells. A large body of data implicates tyrosine kinases as critical components of these signaling pathways. This article focuses on recent genetic evidence that demonstrates a role for selected tyrosine kinases in B lymphopoiesis. We do not provide an exhaustive review but present representative examples of the effects of gain and loss of function mutants

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of tyrosine kinases and their regulators on B cell development and function. These include naturally occurring mutations in mice and humans, knockout and transgenic mice, and manipulation of tyrosine kinase expression in tissue culture models.

B Cell Development

B cell development occurs primarily in the fetal liver and adult bone marrow. Fetal and adult stem cells differ in their ability to give rise to B lineage cells (1). While adult stem cells predominantly develop into conventional B cells, fetal liver stem cells can also differentiate into a unique subset called B-1 cells. B-1 cells persist in the adult animal, are resident primarily in the peritoneum, and express the CD5 antigen. They have a repetoire, distinct from that of conventional B cells, which includes a relatively high proportion of autoantibodies.

The initial, antigen independent stages of conventional B cell development in the adult occur in the bone marrow in response to stromal cell contact and cytokines. Stages of early B cell differentiation have been defined according to alternative classifications (2–5). For this review, we use the system of Hardy et al (4) (Figure 1), which resolves stages of B cell development according to differential expression of cell surface markers.

All cells in the B lineage express the cell surface marker B220. Pro-B cells, which consist of fractions A, B, and C as defined by Hardy, also express CD43. Fraction A represents the earliest identifiable cell type committed to the B lineage. These cells are dependent on stromal contact for growth and are not responsive to IL-7. They retain their Ig genes in germline configuration. Cells in fraction B have upregulated expression of heat stable antigen (HSA), $\lambda 5$, V pre-B, Igα, and Igβ. They have DJ rearrangements and grow in response to IL-7 and stroma. VDJ rearrangements have occurred to a significant degree in fraction C. These cells express both HSA and BSP-1 in addition to $\lambda 5$, V pre-B, Igα, and Igβ. These cells are responsive to IL-7 in the absence of stroma. Greater than 30% of these cells are cycling in adult bone marrow. Fraction D is marked by the downregulation of CD43 expression. Development of these small resting pre-B cells requires membrane-bound Ig heavy chain (6), $\lambda 5$ (7), and IL-7 (8, 9). Production of immature B cells (fraction E) proceeds with light chain rearrangement and the appearance of IgM on the cell surface (sIgM). Immature B cells leave the bone marrow and migrate to the periphery where self-reactive cells are deleted or anergized. This process can also occur in the bone marrow.

The remaining, antigen dependent stages of B cell development occur in the periphery. When mature B cells encounter antigen in the context of T cell help, they are activated to proliferate and differentiate into either antibody secreting plasma cells or memory B cells. Somatic hypermutation of Ig genes occurs at

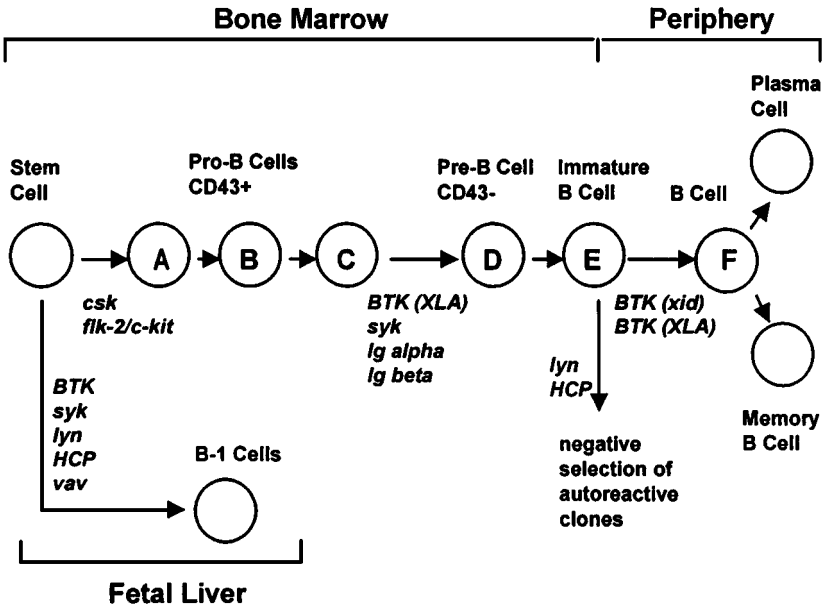

Figure 1 Schematic diagram of B cell development (4). The stages of development affected by loss of function mutations of the tyrosine kinases, phosphatases, and their substrates discussed in this review are indicated in italics. All mutations except HCP result in partial or complete blocks in development. Lack of HCP results in increased numbers of B-1 cells and a reduced threshold for negative selection.

this point to generate antibodies with higher affinity for antigen. In response to various cytokines, mature B cells also undergo Ig class switching to produce antibodies with different effector functions.

Biochemical Evidence Implicates Tyrosine Kinases in B Cell Development

The process of B cell development described above is regulated by the stimulation of multiple cell surface molecules by extracellular ligands. Activation of tyrosine kinases is one of the earliest events in response to signaling via cell surface receptors. A large body of in vitro and biochemical evidence suggests that many of the tyrosine kinases and their regulators expressed in B cells (Table 1) play an important role in transmitting the complex signals regulating B lymphopoiesis. This has been reviewed extensively elsewhere and is briefly summarized here. Ligands for the receptor tyrosine kinases c-kit (10–12), flk-2 (13), and IGF-1R (14, 15) can synergize with IL-7 to induce proliferation of

Table 1 Tryosine kinases and phosphatases expressed in B cells

	Family	Kinase or Phosphatase
Kinase	receptor	c-kit, flk-2, IGF-1R, CSF-1R
	src	lyn, fyn, lck, blk, fgr, hck
	Btk	Btk, tec, BMX (?)
	syk	syk
	Jak	Jak1, Jak2, Jak3, Tyk2
	abl	abl, arg
	csk	csk
Phosphatases	receptor	CD45
	non-receptor	HCP, syp

pre-B cells in culture. Cross-linking of various cell surface molecules on B cells, including sIgM, CD19, CD40, and many cytokine receptors, results in the activation of subsets of the src, Btk, syk, and Jak family kinases (reviewed in 16–29). Many of these kinases are physically associated with the receptors either constitutively or upon stimulation by ligand. The importance of tyrosine kinases in transmitting signals from many of these receptors is implied by the ability of protein kinase inhibitors to block these signals (reviewed in 16, 18, 19, 21, 25, 26).

The majority of these observations are correlative and therefore do not demonstrate directly that tyrosine kinases play an important role in B cell development. In addition, many of the events regulating B cell development in vivo are not accurately represented by the in vitro assays used to investigate signal transduction. Loss of function mutants of several tyrosine kinases and phosphatases, either naturally occurring or engineered by homologous recombination, have provided animal models with which to study more definitively the role of tyrosine phosphorylation in B cell development. These experiments have revealed both unique and redundant functions for tyrosine kinases in this process.

DIRECT GENETIC EVIDENCE FOR REGULATION OF B CELL DEVELOPMENT BY TYROSINE KINASES: Redundancy vs Unique Function

The Receptor Tyrosine Kinases flk-2 and c-kit Play a Role Early in B Lymphopoiesis

The power of a genetic approach for addressing the issue of redundancy of related tyrosine kinases with similar patterns of expression and in vitro functions

is illustrated by a comparison of the B cell phenotypes of mice deficient for expression of the receptor tyrosine kinases c-kit and flk-2. Both are members of the PDGF-R superfamily of receptor kinases (30–32). They are both expressed in progenitor cells of all hematopoietic lineages (32–34). In vitro studies demonstrate that ligands for both these receptors can synergize with IL-7 or each other to promote pre-B cell growth, but neither can do so on its own (10–13). Flk-2 ligand but not SLF (the c-kit ligand) can weakly stimulate production of $B220^+$ cells from $B220^-$ progenitors in culture (13).

Despite the stimulation of B cell growth by SLF in vitro, little effect on B cell development is observed in vivo in the absence of c-kit or SLF. The naturally occurring mouse mutations *W* and *Sl* have deficient function of c-kit (35, 36) and SLF (37–40), respectively. These mice are characterized by defects in hematopoiesis, melanogenesis, and gametogenesis (41). *W* mice have normal pre-B, immature B, and mature B cell populations (42), as do mice that have been injected with the anti-c-kit mAb ACK2 (33). In addition, stroma derived from *Sl* mice can support the growth of pro-B cells, indicating that SLF is not required for early B cell development (43). There may be a mild intrinsic defect in B cell progenitors in *W* mice, as they are less efficient than wild-type progenitors in generating LPS-responsive, mature B cells 10 days after bone marrow transplant into irradiated recipients (42). This deficiency is compensated for during steady state hematopoiesis, however, as B cell numbers are normal in c-kit mutant mice.

These observations suggest that c-kit and flk-2 serve redundant functions. The phenotype of flk-2 knockout mice indicates that while this is true to a significant degree, flk-2 has some unique functions in B cell development (44). These mice have 50% normal levels of pro-B cells and variable numbers of pre-B cells in their bone marrow. They also have a two- to sixfold decrease in the number of progenitors that can form colonies in agar in response to SLF and IL-7. FACS-sorted stem cells from normal mice can give rise to both myeloid and B lineage colonies when grown on a cloned stromal line. Flk-2–deficient stem cells can form myeloid but not B lineage colonies in this assay. In contrast to the effect seen on early B cell development, normal numbers of immature and mature B cells are observed in the bone marrow and periphery of flk-2 knockout mice. This indicates the presence of mechanisms that compensate for the decreased numbers of early B lineage cells in these animals. The lack of a complete block in B cell development and the absence of any effect on myeloid differentiation in flk-2 knockout mice together suggest partial redundancy of flk-2 function with another mechanism, perhaps c-kit.

Severe defects in hematopoiesis in mice mutant in both flk-2 and c-kit demonstrate that this is indeed the case (44). Fewer than expected numbers of these

animals survive to birth, and those that do die by six weeks of age. Significant decreases in the cellularity of all hematopoietic lineages are apparent. There is a twofold decrease in the frequency of both pro-B and pre-B cells in bone marrow relative to flk-2 knockout mice as measured by FACS. A tenfold drop in the frequency of colonies formed in response to IL-7 and SLF is also observed, in comparison to flk-2–deficient mice. In contrast, although the absolute number of myeloid progenitors is lower than normal in these mice, there is no change in the frequency of these cells. This indicates that in addition to a role in stem cells, flk-2 and c-kit have a specific function in B lymphopoiesis.

Nonreceptor Tyrosine Kinases Have Both Unique and Redundant Functions in B Cell Development

Genetic analysis has also revealed a critical role for nonreceptor tyrosine kinases of the Btk and syk families in B cell development. This approach has confirmed the functional redundancy of several src family kinases that had been suggested by their similar expression and activation patterns in B cells.

BTK IS A CRITICAL COMPONENT OF MULTIPLE B CELL SIGNALING PATHWAYS Mutations in Bruton's tyrosine kinase (Btk) are the genetic basis of the human and murine immunodeficiencies X-linked agammaglobulinemia (XLA) (45, 46) and X-linked immunodeficiency (xid) (47, 48), respectively. Btk is a member of a new subfamily of nonreceptor tyrosine kinases characterized by an N-terminal pleckstrin homology (PH) domain, a proline rich region, and SH3, SH2, and SH1 domains (45, 46). These kinases lack the C-terminal negative regulatory tyrosine and the N-terminal myristylation site present in src family kinases. Btk is expressed at all stages of B cell development except plasma cells (49, 50). Other members of this family may be expressed early during B lymphopoiesis: tec is expressed in a pro-B cell line (51), and BMX is found in bone marrow but not spleen, suggesting that it may be expressed in progenitor cells (52).

The phenotypes of XLA, *xid*, and Btk knockout mice reveal a role for Btk in multiple signaling pathways at various stages of B cell development and function. XLA patients have less than 1% of mature B cell numbers and a severe deficit of circulating Ig (53) due to a block at the pro-B–pre-B transition (54). Nonrandom X chromosome inactivation in B cells of female carriers demonstrates that this defect is intrinsic to the B lineage (55). This phenotype can be caused by point mutations or deletions in all subdomains of Btk, indicating that multiple protein/protein interactions are required for Btk function (46, 56–66).

A point mutation in the PH domain of Btk, R28C (47, 48) results in a more mild phenotype in *xid* mice (reviewed in (67, 68). These animals have 30–50% normal levels of conventional B cells and no B-1 cells (67, 68). The B cells present have an immature phenotype (67, 68) and defective signaling in

response to LPS (67, 68), IL-5 (69, 70), and IL-10 (71), or to cross-linking of sIgM (67, 68), CD38 (72, 73), and CD40 (74). *xid* mice cannot respond to type II T-independent antigens, and they produce decreased levels of total IgM and IgG3 (67, 68).

Surprisingly, the phenotype of Btk knockout mice is much more similar to *xid* than to XLA. Kerner et al (75) have studied the ability of Btk-deficient ES cells to contribute to the B lineage in chimeric animals. In chimeras with blastocysts derived from Rag 2–deficient mice (76), which do not produce B or T lymphocytes themselves, $Btk^{-/-}$ ES cells exhibit an *xid* phenotype. They produce normal numbers of T cells but reduced numbers of B cells, which cannot respond to type II T-independent antigens. In chimeras with wild-type ES cells, Btk-deficient ES cells demonstrate a competitive disadvantage at the pre-B cell stage. This is in contrast to *xid* cells, which develop normally through the pre-B cell stage, indicating that the *xid* mutation is not completely null. Germline Btk knockout mice have been generated using two independent targeting constructs (77). Both mice are indistinguishable from *xid* mice with respect to B cell populations measured by FACS, Ig levels, response to T-independent antigens, and proliferation in response to cross-linking of sIgM, sIgD, CD40, and LPS. A slight increase in the ratio of pro-B to pre-B cells was observed in both Btk knockout and *xid* mice, suggesting that Btk may also play a minor role early in murine B cell development.

These results clearly illustrate the decreased severity of a Btk null mutant phenotype in mice relative to humans. In addition, an R28H mutation at the same amino acid affected in *xid* mice causes severe XLA (61). Perhaps mice have a mechanism lacking in humans that can compensate to some degree for Btk deficiency. This may be T cell dependent, as *xid/nu* mice have a more substantial block in B lymphopoiesis (78). Another possibility is that the phenotype is dependent on genetic background. This is supported by the observation that there is some variation in the severity of immune deficiency that is dependent on the genetic background of *xid* mice. The *xid* phenotype is more severe on the C3H/HeN background relative to CBA/N (79). B cells do not respond to type I T independent antigens or to several B cell mitogens. They also have a more immature cell surface phenotype (79). In contrast, C57BL/6.*xid* mice have increased numbers of periperal conventional B cells and peritoneal B-1 cells relative to CBA/N *xid* animals (77).

Biochemical as well as genetic evidence supports a role for Btk in multiple B cell signaling pathways. Btk is activated in response to IL-5 (80), IL-6 (51), and sIgM cross-linking (81–84). This occurs late relative to the activation of src family kinases (83), suggesting that Btk may by regulated by src family kinases. This hypothesis is supported by two lines of evidence. The proline-

rich domain of Btk can bind to the SH3 domains of the src family kinases lyn, fyn, and hck in the yeast two-hybrid system (85). Overexpression of both lyn and Btk in fibroblasts leads to both transphosphorylation of Btk at Y551 and autophosphorylation of Btk at an unidentified tyrosine residue, resulting in increased Btk kinase activity (D. Rawlings, A. Scharenberg, H. Park, S. Lin, R. Kato, O. Witte, and J-P. Kinet, unpublished observations). Additional protein/protein interactions have also been described for Btk. The PH domain can interact with the β/γ subunits of heterotrimeric G proteins (86) and protein kinase C (87).

Gain of function mutants have been useful both in ordering components of a specific signaling pathway and in identifying pathways in which a particular gene is involved. Btk* is a gain of function mutant isolated in a random mutagenesis screen for alleles that could transform NIH-3T3 cells (88). It carries a point mutation in the PH domain that results in constitutive phosphotyrosine deposition and increased membrane localization. This allele of Btk can weakly relieve the IL-5 dependence of a pro-B cell line, solidifying the genetic and biochemical evidence that places Btk in an IL-5 signaling pathway. However, Btk* does not by itself transform hematopoietic cells. This will be a useful tool to dissect signal transduction pathways involving Btk in cell lines. This approach has already been used to show that Y551 and an intact PH domain are required for downstream signaling by Btk* in NIH-3T3 cells. Btk* will also be useful to illuminate additional roles for Btk in vivo using transgenic mice.

SYK IS ESSENTIAL FOR PRE–B CELL DEVELOPMENT Both genetic and biochemical evidence indicate that syk also plays a critical role in B cell development. Syk (89) is a member of a small subfamily of nonreceptor kinases, which also includes the T cell–specific kinase ZAP/70 (90). These kinases have two SH2 domains and an SH1 domain, and they lack the C-terminal negative regulatory tyrosine found in src family kinases. ZAP/70 deficiency in humans due to destabilizing mutations results in nonfunctional $CD4^+$ T cells and a lack of $CD8^+$ T cells (91, 92), suggesting that this family is important for lymphopoiesis.

Several experiments demonstrate that syk is important for signaling from sIgM. Syk has been shown to interact physically with sIgM in two ways. It can bind to tyrosine phosphorylated Igα and β (components of the sIgM signaling complex) via its SH2 domain after Ig cross-linking (93–95), and it can associate constitutively with sIgM in the absence of Igα and β (96). Cross-linking of sIgM activates syk (83, 95–97). This does not occur in a lyn-deficient chicken B cell line (98), indicating that phosphorylation of syk by src family kinases is required for its activation. Activation of syk per se is necessary but not

sufficient for sIgM signaling. A csk-deficient B cell line that has constitutively active syk still requires sIgM cross-linking for downstream signaling events to occur (99). However, IP^3 generation and Ca^{++} flux do not occur in response to sIgM cross-linking in a syk-deficient B cell line (100). This defect can be rescued by transfection of the cells with ZAP/70 but not with a syk allele with a mutated SH2 domain (94). The activation of syk in response to IL-2 (101) and changes in phosphorylation and subcellular distribution of syk after CD40 cross-linking (102) indicate that syk plays a role in other B cell signaling pathways as well.

As predicted from the above observations, analysis of syk knockout mice shows a critical function for syk in B cell development in vivo (A. Cheng, A. Pawson, personal communication). Lack of syk results in perinatal lethality due to excessive hemorrhage during embryogenesis. However, the use of chimeras with Rag $2^{-/-}$ blastocysts (76) and the transplantation of fetal liver stem cells from $syk^{-/-}$ mice into $Rag2^{-/-}$ recipients allow the study of lymphopoiesis in the absence of syk. While T cell development is normal, no $B220^+$ IgM^+ B cells are found in spleen and bone marrow. B cell differentiation appears to be blocked at the transition from $CD43^+$ to CD43− cells in the bone marrow. B cells in mice deficient in λ5 (7), membrane-bound Ig heavy chain (6), and IL-7R (9) also fail to develop beyond the pro-B stage. This suggests that syk is a downstream component of one or more essential signals for B cell development that emanate from these surface molecules. Syk is also required for the development of B-1 cells (A. Cheng, A. Pawson, personal communication).

SRC FAMILY KINASES HAVE BOTH OVERLAPPING AND UNIQUE FUNCTIONS IN B CELLS The activation of src family tyrosine kinases in response to signals important for B cell development and their ability to phosphorylate Btk and syk, both of which are critical for B lymphopoiesis, suggest that they too play an important role in this process. Six members of the src family—lyn, fyn, lck, blk, fgr, and hck—are expressed in B lineage cells. All except hck have been shown to be activated in response to sIgM cross-linking (103, 104), reviewed in (16–24). Overlapping subsets of these are also activated by stimulation of CD40 (105), CD19 (106), IL2-R (27, 29), and IL-7R (107). The significant redundancy among src family members suggested by these observations is illustrated by the B cell developmental phenotypes of several src family–deficient mice.

Knockouts of lyn (M. Hibbs, A. Dunn, personal communication), lck (108), fyn (109, 110), fgr (111), hck (111), and double knockouts of fyn/yes (112) and fgr/hck (111) have been described. As measured by FACS analysis, conventional B cell development is not impaired in any of these mice. There are several explanations for this result. Perhaps multiple src family kinases can

mediate the signals regulating B cell development. Another possibility is that blk, which has not yet been targeted, plays a dominant role in B lymphopoiesis. Finally, B cell developmental signals may be much more dependent on syk and Btk than on src family kinases.

A dominant role for lyn in sIgM signaling and B-1 cell development In contrast to the apparent lack of requirement for src family kinases in conventional B cell development, a dominant role for lyn in B-1 cell development and sIgM signaling is illustrated by lyn knockout mice (M. Hibbs, A. Dunn, personal communication). B-1 cell development is completely blocked in these animals. There is also a significant decrease in the number of phenotypically mature B cells, most likely due to an inability of the cells to proliferate in response to antigen, as sIgM signaling is impaired in lyn-deficient splenic B cells. Similarly, the lyn gene was knocked out in a chicken B cell line that is particularly amenable to gene targeting by homologous recombination and expresses no other src family kinases (100). Ca^{++} flux and syk activation in response to sIgM cross-linking were abrogated in these cells. These defects could be rescued by transfection of the cells with lyn, fyn, and lck, but not src. As $lyn^{-/-}$ murine B cells should express fyn and lck, this indicates a dominant role for lyn relative to other src family kinases in murine but not chicken B cells. Lyn is also required for LPS but not CD40 signaling (M. Hibbs, A. Dunn, personal communication).

Lyn knockout mice also have defects in immune function (M. Hibbs, A. Dunn, personal communication). Increased levels of IgM are observed as a result of an increase in the number of antibody secreting cells. Responses to both T-dependent and T-independent antigens are impaired. These animals also suffer from autoimmune disease characterized by circulating autoantibodies and Ig deposits in the kidneys. This suggests that lyn is required for tolerance induction.

IL-5 signaling is mediated by fyn A dominant role for lyn in sIgM signaling is confirmed by the observation that splenic B cells lacking fyn respond normally to sIgM cross-linking (113, 114). However, IL-5 response is completely dependent on fyn (114). $Fyn^{-/-}$ mice also have slightly impaired response to T-independent antigens (114). Increased levels of IgM and IgG2a have been reported in fyn-deficient mice (109), but this phenotype has not been observed consistently (114).

These results demonstrate that individual effector functions of mature B cells are dependent on specific src family tyrosine kinases. As *xid* mice have deficient responses to all of these signals, it is attractive to hypothesize that Btk is at the convergence of several pathways mediated by individual src family kinases.

INDIRECT GENETIC EVIDENCE FOR REGULATION OF B CELL DEVELOPMENT BY NONRECEPTOR TYROSINE KINASES

Despite the inability to uncover a function for src family kinases in B cell development using knockouts of the kinases themselves, a growing body of genetic evidence demonstrates the importance of their substrates and regulators in B lymphopoiesis. These data also confirm the role of src family kinases in sIgM signaling in immature and mature B cells.

Tyrosine Kinase Substrates Are Key Components of B Cell Signaling Pathways

TYROSINE PHOSPHORYLATION OF IG-α AND β IS REQUIRED FOR EARLY B CELL DEVELOPMENT Igα and β are key signaling components of the sIgM complex(115). In resting cells, src family members are preferentially associated with Igα (116, 117). Upon sIgM cross-linking, the ARH1 motifs of Igα and β are tyrosine phosphorylated (22, 118, 119). ARH1 domains consist of the sequence (D/E)XXYXXL$(X)^{6-8}$YXXL and are conserved among several antigen receptor signaling components (120). This phosphorylation results in increased association of src family members (121) and syk (95) with the sIgM signaling complex as well as in their activation. Mutations in both ARH1 tyrosines abrogate signaling through the Igα and β cytoplasmic domains in cell lines (119, 122, 123). The block in B cell development observed in membrane-bound Ig heavy chain (6) and λ5 (7) knockout mice suggests that signaling through a "pre-B receptor" which also contains Igα and β is required for B lymphopoiesis.

To investigate the role of phosphorylation of the Igα ARH1 motif in vivo, Torres et al took the following approach (R. Torres, K. Rajewsky, personal communication). They knocked out one endogenous Igα allele and replaced the other with an Igα gene carrying mutations in both ARH1 tyrosines using homologous recombination in ES cells. Mice derived from these cells express only the mutant Igα protein, and they have a severe block in B cell development. Normal levels of $CD43^+$ pro-B cells (Fractions A-C) are present, but decreased numbers of pre-B and immature B cells are observed in bone marrow. There are only 1–3% of normal levels of peripheral B cells.

Interaction between Ig heavy chain and Igα and β is required for the pro-B–to–pre-B transition (124). To investigate the mechanism by which Igβ mediates this event, the following approach was taken. A fusion protein between the extracellular and transmembrane domains of the Ig heavy chain and the cytoplasmic tail of Igβ is able to transmit signals that mimic those of an intact sIg complex (115, 123). When introduced as a transgene, this chimeric molecule was able to partially rescue the phenotype of Rag-1 deficient mice

(124). Pre B–cell development and allelic exclusion were induced by the transgene. Rag-1 knockout mice carrying a similar transgene with mutations in both ARH1 tyrosines were indistinguishable from nontransgenic littermates. This demonstrates that tyrosine phosphorylation of the Igβ ARH1 motif can drive the pro-B–to–pre-B transition.

These experiments indicate that phosphorylation at the ARH1 tyrosines of both Igα and Igβ is necessary for proper differentiation of pre-B cells in vivo. This strongly suggests that the function of src family kinases is required for early B cell development.

VAV IS REQUIRED FOR SIGM SIGNALING AND B-1 CELL DEVELOPMENT The proto-oncogene vav is tyrosine phosphorylated in response to sIgM cross-linking (125–127). To address the role of vav in B lymphopoiesis, B cells derived from wild-type and vav$^{-/-}$ ES cells in Rag 2$^{-/-}$ chimeras were compared (128, 129). Variable decreases in the numbers of peripheral B cells were observed in vav$^{-/-}$ animals, perhaps due to varying degrees of chimerism. Vav$^{-/-}$ B cells displayed a competitive disadvantage relative to wild-type cells in chimeras with wild-type blastocysts (129). B-1 cell development was completely inhibited in the absence of vav (128, 129). B cell proliferation in response to sIgM but not CD40L or LPS requires vav (128, 129). The developmental defects observed here are similar to those in Btk-deficient mice (75, 77). However, the requirement of Btk for CD40 and LPS signaling indicates that Btk is involved in vav independent pathways as well. Whether tyrosine phosphorylation of vav per se is required for its function in vivo has yet to be determined.

The Regulatory Tyrosine Kinase csk Is Essential for Early Lymphoid Development

Additional evidence for a role for src family kinases in lymphopoiesis comes from the study of the regulatory tyrosine kinase csk. Expressed primarily in thymus and spleen, csk phosphorylates the C-terminal negative regulatory tyrosine of src family kinases (130–132). Mice deficient in csk have increased constitutive activation of src family kinases and die during embryogenesis due to defects in neurulation (133, 134). In chimeras with wild-type or Rag 2$^{-/-}$ blastocysts, csk$^{-/-}$ cells were able to contribute to heart, brain, liver, kidney, and bone marrow myeloid progenitors and splenic myeloid cells, but not to either lymphoid lineage in adults (135). This preferential block in lymphoid development in the absence of csk implies that src family kinases play a negative role early in lymphopoiesis, in addition to any positive role they may have in later stage B cells, as suggested by biochemical experiments.

While the src family kinase lyn is necessary for sIgM signaling (100), (M. Hibbs, A. Dunn personal communication), studies with csk$^{-/-}$ chicken

B cell lines demonstrate that constitutive activation of src family kinases is not sufficient for this process (99). Inactivation of a protein tyrosine phosphatase may also be required, as overall tyrosine phosphorylation is not dramatically increased in csk$^{-/-}$ B cells (99).

Protein Tyrosine Phosphatases

Regulation of signal transduction by phosphorylation is a dynamic process that also requires dephosphorylation of kinase substrates. The phenotypes of mice deficient in the expression of the protein tyrosine phosphatases CD45 and HCP stress that the interplay between tyrosine kinases and phosphatases is important for B cell development and function.

CD45 IS NECESSARY FOR SIGM SIGNALING BUT NOT B CELL DEVELOPMENT CD45 is a transmembrane tyrosine phosphatase expressed as cell type–specific isoforms throughout the hematopoietic system. It can dephosphorylate the negative regulatory tyrosine of src family kinases (136–138) as well as other substrates including Igα and β (139). It can be coimmunoprecipitated from B cells with lyn and Igα and β (140). CD45-deficient B cell lines have impaired sIgM signaling (17, 139, 141, 142). Similarly, B cells from CD45 knockout mice proliferate in response to LPS but not sIgM (143). This suggests that activation of src family kinases by C-terminal dephosphorylation is necessary for signaling through sIgM. B cell development in CD45$^{-/-}$ mice is normal (143). This indicates that CD45 either is not required for signaling through Igα and β at early stages or is redundant with another phosphatase early in development.

HCP/PTP-1C NEGATIVELY REGULATES SIGM SIGNALING Mutations in the cytoplasmic tyrosine phosphatase HCP (also known as PTP-1C) are the genetic basis of the *motheaten* (*me*) phenotype in mice (144–146). These animals have severe defects in all hematopoietic lineages. They have elevated numbers of macrophages, granulocytes, and erythroid cells, and they also have reduced NK and T cell activity (147). They suffer simultaneously from immune deficiency and autoimmunity in that they lack mature conventional B cells yet develop high levels of autoantibodies by one month of age (148–151). Increased numbers of B-1 cells are most likely responsible for the production of these autoantibodies.

Overgrowth of *me* myeloid cells can suppress B lymphopoiesis in *trans* in both in vitro culture systems (152, 153) and mixed bone marrow chimeras (154). This has made it difficult to determine whether the B cell defects in *me* mice are cell autonomous. Titration of *me* cells in mixed bone marrow chimeras has recently been used to demonstrate that a B cell phenotype is still observed in the absence of any *trans* inhibitory effect of myeloid cells (154). While expected numbers of *me*-derived immature B cells were present in the bone marrow of

these mice, there was a tenfold decrease in the number of *me* peripheral B cells. Those B cells that were retained had characteristics similar to anergic B cells even in the absence of antigen.

To investigate the role of HCP in negative selection of B lymphocytes, the *me* mutation was crossed into the anti-hen egg lysozyme (HEL) Ig transgenic system. The majority of B cells in anti-HEL Ig transgenic mice express anti-HEL antibodies. When these B cells are exposed to HEL, deletion or anergy occurs depending on the valency of the antigen (155). The *me* mutation resulted in a reduced threshold for the deletion of self-reactive B cells (154). *me* B cells are hypersensitive to sIgM cross-linking as measured by Ca^{++} flux (154) and proliferation (156). It has also been shown that HCP is required for Fc-γ RIIBI inhibition of sIgM signaling (156, 157). These data demonstrate that HCP negatively regulates sIgM signaling and implicate tyrosine kinases as positive regulators of this process.

The mechanism by which HCP downregulates Ig signaling is not known, although Btk, Jak, and src family kinases may all be involved. Crossing the *xid* mutation onto the *me* background blocks autoantibody production and B-1 cell expansion (158). This suggests that Btk directly counteracts HCP or is downstream of and necessary for signaling by kinases that do. HCP can bind to tyrosine phosphorylated cytokine receptors via its SH2 domain. An EPO receptor mutant that cannot bind HCP induces increased activation of Jak2, indicating that HCP negatively regulates Jak2 (159). Consistent with this observation, HCP can dephosphorylate and inactivate Jaks in vitro (28).

HCP may also serve to prevent sIgM signaling in resting cells. HCP can bind to sIgM, and this interaction is disrupted upon sIgM cross-linking (156). In addition, a 35-kDa component of the sIgM complex, believed to be Igα, can be dephosphorylated by HCP (156). This suggests a model whereby HCP associates with and dephosphorylates Igα and β in resting B cells. Upon sIgM cross-linking, HCP is released, allowing phosphorylation of Igα and β by src family kinases. This phosphorylation is most likely mediated by lyn as demonstrated by the requirement for lyn in sIgM induced proliferation and negative selection (M. Hibbs, A. Dunn, personal communication). Signaling may subsequently be terminated by reassociation of HCP with the complex.

CONCLUSIONS AND FUTURE DIRECTIONS

The data presented in this review demonstrate the importance of reversible tyrosine phosphorylation at multiple stages of B cell development and function (Figure 1, Table 2). These include the pro-B–to–pre-B transition, antigen-induced B cell tolerance, activation of mature B cells, signaling in response to numerous cytokines and B cell coreceptors, and development of B-1 cells. Both

Table 2 Regulation of B cell signaling pathways by protein tyrosine kinases and phosphatases

Receptor or stimulus	Enzyme or substrate required for signaling
sIgM	Btk, syk, lyn, vav, Ig alpha, Ig beta, CD45 HCP (downregulates signaling)
LPS	Btk, lyn
IL-5	Btk, fyn
IL-10	Btk
CD38	Btk
CD40	Btk

unique and redundant functions for different kinase families in these processes have been revealed by genetic analysis. Dominant roles are played by Btk, syk, and lyn. The next step on the road to further understanding the role of tyrosine kinases in B cell development is to use genetic systems to address questions of redundancy as well as to delineate and order components within a specific tyrosine kinase–dependent signaling pathway.

There are several tyrosine kinases expressed in B cells—including blk, IGF-1R, and all four Jak family members—for which knockout mice have not yet been reported. The phenotypes of these animals may either reveal a role for these genes in B lymphopoiesis or demonstrate that these kinases are redundant. Multiple Jak kinases are expressed in B cells, suggesting that some degree of redundancy will be observed when mice deficient in these genes are generated. The creation of animals deficient in multiple members of each family should elucidate the role of that group of kinases in B lymphopoiesis.

Constitutively active forms of tyrosine kinases such as Btk* (88) or src family kinases mutated at the C-terminal negative regulatory tyrosine will be invaluable genetic tools. Transgenic mice expressing these alleles in B cells can be examined for alterations in development or in specific signaling pathways. This approach has been used successfully for lck (160–162) and fyn (163) in T cells. These transgenic mice can also be crossed with mice deficient in other B cell signaling molecules to place the various components of a pathway in order. Many knockouts of cell surface molecules or their ligands affect different stages of B cell development and function, including λ5 (7), membrane-bound Ig heavy chain (6), CD40 (164), CD40L (165), IL-7 (8), IL7-R (9), and IL2-R gamma (166). Stimulation of these receptors activates multiple nonreceptor tyrosine kinases, but it is not clear which of these kinases are sufficient to transmit the signal. In addition, the phenotypes of loss of function of Btk and syk are similar to many of these receptor knockouts, implying that they are downstream of these receptors. The ability of an activated kinase transgene to rescue the

defects in B cell development in any of these knockouts would demonstrate that the two genes are in the same signaling pathway.

The concept of rescuing a knockout phenotype with a downstream transgene can also be applied to looking at the role of tyrosine kinase substrates in a manner similar to the experiments with Igα and β described above. If a knockout of a kinase substrate such as vav (128, 129) has an effect on B cell development or signaling, the inability of a mutant allele of this gene that cannot be tyrosine phosphorylated to rescue this defect would demonstrate that tyrosine phosphorylation of that protein is required for signaling.

The recent development of the cre-lox system for cell type–specific gene targeting (167) will also be useful for the study of the function of tyrosine kinases in B cell development. First, mice deficient in some kinases or phosphatases such as syk, csk, and HCP are either embryonic lethal or have phenotypes in lineages other than B cells. A B cell–specific knockout of these genes would prove that any B cell defect observed is cell autonomous and thus would avoid the need to use chimeric animals. Secondly, kinases such as Btk and syk appear to be involved both in early B cell development and late in the function of mature B cells. A knockout of these genes in mature B cells only would provide a system whereby sufficient numbers of mature cells would be generated in the absence of a developmental block such that effects of the mutation on signaling could be easily studied. A limitation of this approach that must be considered, however, is that the targeted gene is not deleted in all the cells of interest (167).

The examples provided here demonstrate that mutations in tyrosine kinases and their regulators can result in immune deficiency or autoimmunity. This suggests that a more complete understanding of how reversible tyrosine phosphorylation influences normal B lymphopoiesis may lead to the development of therapies for diseases of the immune system.

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ORCHESTRATED INFORMATION TRANSFER UNDERLYING LEUKOCYTE ENDOTHELIAL INTERACTIONS[1]

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ABSTRACT

The specificity and efficiency of leukocyte binding to endothelial cells (ECs) depends on coordinated information transfer from the underlying tissue to endothelium and from there to the leukocyte. We address three distinct information-transfer points in this system: 1. How does the leukocyte read information from the EC? This process is best accounted for by the paradigm of a multi-step adhesion cascade optimized for rapid information readout; it consists of primary adhesion (rolling/tethering), triggering, and strong adhesion. Recent studies with T cells, monocytes, and eosinophils confirm the generality of the paradigm. The concept of primary adhesion has been expanded to involve not only the selectins, but also certain integrins; furthermore, it depends on receptor concentration on leukocyte microvilli. 2. What information from the underlying tissue does the EC transform into signals for the leukocytes? And what rules govern that process? We illustrate the principles with chemokines, believed to participate in the triggering

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step. The endothelium displays chemokines either (*a*) directly by "posting" them from other cells or (*b*) by integrating a variety of tissue and environmental signals and "relaying" that information by producing its own chemokines and surface adhesion molecules. The rules for this endothelial transduction include specificity coupled with redundancy, amplification, synergy, and coordinated induction of ensembles of molecules. Finally, 3. How does the relevant information reach the endothelium? Simple diffusion is sufficient to deliver signals from cells close to the vessel. However, longer range soluble mediator transport appears to be facilitated by fiber bundles, particularly those ensheathed by fibroblastic reticular cells in the lymph node.

INTRODUCTION—THE INFORMATION PROBLEM

Leukocyte-endothelial interactions are a special case of cell sorting, in which the endothelium discriminates among circulating leukocytes in order to select cells for transmigration into surrounding tissue. Unlike cell sorting in embryogenesis or wound healing, where contact between cells lasts minutes to hours, sorting in the vascular system depends on contact that lasts much less than a second unless specific adhesive events prolong it. The biological objective of leukocyte-endothelial interactions is to direct circulating cells into their appropriate tissue sites with efficiency and specificity. Endothelial cells (ECs) play a singular role in this process, receiving information from the underlying tissue and transforming it into information that can be read rapidly by the passing leukocyte.

There is a high degree of specificity in the interaction of ECs with circulating cells. "Recirculation" is the process whereby lymphocytes undergo repetitive cycles of migration from the circulatory system into tissue and back into the vasculature. Regardless of where a foreign antigen enters the body, this process assures that it will be found by an immune cell with the relevant antigen-specific receptor ("immune surveillance"). Thus, T cells, B cells, and NK cells all undergo continuous recirculation, even in the absence of injury or inflammation. Recirculation is governed by a high degree of specificity, so that phenotypically distinct subsets of these cells migrate preferentially into certain anatomic sites. Superimposed on this is "recruitment" of immune cells to inflammatory sites. The anatomic location and the nature of the inflammatory stimulus determine which leukocytes migrate to an inflammatory site; usually recruitment includes cells that do not recirculate such as neutrophils, eosinophils, and monocytes. In addition to these commonly cited roles of mature cells in immune responses, specific migration occurs in other contexts, including odysseys of T cell and B cell precursors throughout their maturation, dispersal of γ-δ T cells to their

relevant tissues, and the seeding of stem cells. In short, we infer that there are dozens of distinct circulating cell types that are discriminated during endothelial interactions (1, 2). In this review we focus on the biological strategies of information transfer, from tissue to endothelium to leukocyte, which make possible this high level of specificity, and we refer readers to other reviews for detailed descriptions of which subsets migrate under particular pathophysiologic conditions, and for more background information on the molecules involved (1, 3–11). Transmigration into tissue subsequent to binding is a complex sequence of events (12) not addressed in this review.

CIRCULATING CELLS "READ" INFORMATION FROM ENDOTHELIUM

A Prototypic Adhesion Cascade

Understanding of the rapid binding events between leukocytes and endothelium has given rise to a conceptually powerful paradigm that views the interaction as a multistep process (1, 3–11). As a starting point, we present a prototypic adhesion cascade: an in vitro model of neutrophil binding to ischemia-injured endothelium described by Nash and coworkers (13). Cultured endothelium within a tube is transiently injured by ischemia and then perfused with a suspension of granulocytes. Granulocytes that flow adjacent to the ECs are captured and begin to roll along the surface. This constitutes the first step of the cascade, called "tethering" or "primary" adhesion. This interaction is mediated by P-selectin on the EC, previously shown to bind the granulocyte mucin PSGL-1 (14). This is consistent with the cascade model, in which selectins mediate tethering. From the point of view of information transfer, this step is arguably the most important. It slows the forward motion of the leukocyte and maintains its surface in close proximity to the surface of the endothelium (see discussion below).

The next *visible* step in the cascade is arrest, in which the rolling granulocyte abruptly becomes immobile. Antibody inhibition studies in the ischemia model demonstrate that arrest is mediated by granulocyte $\beta 2$ integrins, presumably by binding to endothelial ligands ICAM-1 and ICAM-2. This step of the cascade, known as "secondary" or "strong" adhesion, is always mediated by integrins, which efficiently and rapidly regulate adhesion (15).

A Triggering Step Precedes Strong Adhesion

This step is undetected unless correct strategies are used to demonstrate it, and thus its importance is often overlooked. In the ischemia model, antibody to the chemokine IL-8 inhibits arrest by more than 85% (13). Thus, IL-8 produced

by ischemic endothelium is as critical to the process as are either the primary or secondary adhesion pathways. The time of action of IL-8 lies after the rolling (which anti-IL-8 does not inhibit) and before the arrest. Conceptually, triggering is needed to "activate" the integrins; despite high levels of expression on circulating cells, integrins are generally in a functionally inactive state that can be rapidly converted to an active one (15).

The cascade has the potential for encoding an enormous amount of information, as clearly articulated by Butcher (1). Each of the three prototypic steps occurs via specific receptor-ligand combinations. According to the paradigm, strong adhesion will only result efficiently when a leukocyte expressing one set of three receptors meets an EC displaying the complementary set of three counter-receptors. Since each of the receptors and ligands can be selectively expressed on leukocytes and ECs, the number of possible combinations is very large.

Primary Adhesion and Its Roles in Information Readout

The paradigm of the adhesion cascade has been solidified and refined in the last several years. Originally derived from observations in granulocytes (1, 16), it was rapidly generalized to all leukocytes, based on fragmentary information. Validation is now forthcoming for other important circulating cell types. Many studies have distinguished primary and secondary adhesion events for T cell binding to endothelium, either in vitro (17–26) or in vivo (24, 27), as has one study of monocytes in vitro (23) and another of eosinophils in vivo (28).

The findings for each of these are remarkably consistent with the cascade paradigm—although they necessitate a few refinements. Each cell type studied can use selectins for its primary adhesion (rolling). For example, under different experimental conditions, each of the three selectins can mediate rolling of T cells. Those subsets of T cells that roll are those possessing the relevant selectin ligand, including CLA for E-selectin, PSGL-1 for P-selectin, and L-selectin for PNad (peripheral lymph node adressin) (17, 22). However, one notable broadening of the paradigm is that molecules other than selectins can mediate rolling. The integrin molecules $\alpha 4\beta 1$ and $\alpha 4\beta 7$ mediate both rolling (characteristic of primary adhesion) and arrest (21, 24, 28). Indeed, integrins bind to the same ligand for both rolling and arrest (VCAM-1 for $\alpha 4\beta 1$ and principally MAdCAM-1 for $\alpha 4\beta 7$). Integrin-mediated rolling occurs both in vitro, with purified ligand, and in vivo.

There appear to be additional molecular pathways that mediate leukocyte rolling on endothelium. Rolling of human T cells in vitro on IL-1-activated ECs is not inhibitable by anti-selectin or anti-integrin antibodies, suggesting additional receptors and or ligands (20). The binding of CD44 to hyaluronic

acid can mediate rolling and thus may represent another class of receptor-ligand interaction in primary adhesion. For example, the BW5147 cell line rolls either on purified hyaluronate or cultured ECs; in each case the binding is dependent on both CD44 and hyaluronate (29). Although the biologic relevance of this result remains uncertain, it may help explain the involvement of CD44 and hyaluronate in lymph node binding and homing (30, 31).

A second extension of the concept of primary adhesion is that rolling can occur between cells other than leukocytes and EC. Platelets roll along on P-selectin present on activated endothelium (D Wagner, personal communication). Trauma-induced histamine release from mast cells, with resultant endothelial P-selectin externalization, may allow platelet rolling in order to effectively "search out" areas of vessel wall damage. In addition, granulocytes participate in selectin-mediated rolling on immobilized platelets (32) and on granulocytes that have recently adhered to endothelium (33). Thus, at areas of acute inflammation/damage, platelets and granulocytes can act as partial surrogates for the endothelial surface in primary adhesion. Finally, certain carcinoma cells can roll on endothelium (34, 35), although the relevance of this in vivo is unknown. Thus, rolling occurs in a wider range of intravascular binding events than was previously appreciated.

The third extension is the importance of microvilli in primary adhesion. Based on scanning EM studies in the 1970s (36, 37), leukocyte binding to endothelium was postulated to occur via microvilli. The theoretical argument was that close contact over broad membrane areas would be prevented by the electrostatic repulsion of the negatively charged surface glycocalyx present on each cell. Thus, microvilli—thin, long (up to 0.5 μm) protrusions—would more easily make contact because of dramatically reduced net charge. Molecular evidence came from studies demonstrating that L-selectin is preferentially expressed on the tips of granulocyte microvilli (38). The $\alpha 4\beta 7$ integrin that mediates primary adhesion is likewise concentrated on the tips of lymphocyte microvilli (24); in contrast, the LFA-1 integrin does not mediate primary adhesion and is not selectively expressed on microvilli. Compelling validation of this concept comes from studies of the functional effects of changing the spatial localization of L-selectin at the cell surface (132). When the cytoplasmic tail of L-selectin is exchanged for that of CD44, the chimeric molecule now localizes primarily to the cell body rather than to microvilli; such cells no longer roll efficiently on L-selectin ligand under flow, although they still bind under static conditions.

At least three biophysical strategies make efficient rolling possible. First, there are rapid on-rates between receptor and ligand (3, 39). Second, the complementary receptors are spatially distributed to optimize encounter. This

is achieved both by the placement of receptors on the tips of leukocyte microvilli and by using receptors (both selectins and mucins) that are long and stiff, thereby exposing their binding sites beyond the bulk of the glycocalyx (40). Third, local concentrations of receptor/ligand are very high. L-selectin is expressed at a high uniform level of approximately 300,000 molecules per cell on most circulating leukocytes (41, 42), which makes it one of their most abundant surface molecules; when it is concentrated on the tips of microvilli, its effective local concentration will be exceptionally high. The mucin counterreceptors likewise can be present at high concentrations, and their repeating carbohydrate side chains represent a high-density array of ligand; they may even be localized on filopodia of ECs (43). The combination of the foregoing strategies makes possible efficient establishment and maintenance of rolling.

Thus, several strategies are utilized to achieve efficient rolling, suggesting that rolling is pivotal to subsequent information transfer between endothelium and leukocyte. Rolling provides two essential elements: time and proximity. First, it prolongs what would otherwise be a repulsive encounter of a few milliseconds into a period of contact as long as several seconds. Second, it maintains contact between the leukocyte and the information source (the endothelium) for the subsequent events in the cascade. Furthermore, the molar concentrations of cell surface molecules are extremely high because they are constrained to diffuse in the plane of the membrane (44); rolling presumably establishes an optimal separation distance between the cells, thereby increasing the frequency of receptor-ligand encounters.

Insights and Controversies on the Triggering Step

Unlike the general consensus that exists on most issues relating to primary adhesion and secondary adhesion, controversy surrounds the triggering step: First, what agents are proposed to mediate triggering in the cascade? Second, is triggering a necessary step?

There are two classes of molecular interactions proposed to provide triggering in the cascade. The first class consists of soluble factors present at or near the endothelial surface. These include primarily the classical chemoattractants for myeloid cells (fMLP, C5a, LTB4, and PAF) (45) and the family of chemokines, which includes more than 25 members (46–49); all of these signal via serpentine G-protein–linked receptors on the leukocyte (50). An additional soluble factor proposed to trigger the adhesion cascade is HGF, a differentiation factor, capable of acting on a T-lymphocyte tyrosine kinase receptor (51). Of these, so far only IL-8, PAF, and LTB4 have met the rigorous test of triggering leukocyte endothelial adhesion under conditions of flow. Yet the other candidate molecules share many of the properties of the proven mediators—the ability

to induce integrin-mediated adhesion, for example—and therefore are strongly suspected of also mediating triggering. This has been demonstrated for several chemokines and classic chemotactic factors (52–54) as well as for HGF (51). Many also induce chemotaxis, cell polarization, transmigration, and similar responses, which appear closely linked biologically to adhesion triggering in the cascade (46–48). All the foregoing are therefore reasonable candidates for contributing to triggering in the cascade.

Surface molecules are another class of molecules with possible triggering function. L-selectin has been proposed as a trigger, since cross-linking on neutrophils leads to a calcium flux (55, 56), and L-selectin–mediated contact with cultured high endothelial venule (HEV) endothelium triggers motility (57). Although these studies demonstrate that L-selectin transduces some signals, it is unclear whether these are the signals critical for integrin activation since: 1. the calcium flux is relatively slow (40–60; 55); 2. the response is not blocked by pertussis toxin (56; and see below); and 3. L-selectin binding to PNad does not augment LFA-1 binding (17). Activation of leukocyte integrins has also been reported after binding to E-selectin (58, 59) or to CD31 antibody (60, 61).

Is triggering a necessary step in adhesion cascades? Three general kinds of evidence indicate that it is. The most compelling are experiments that identify a required triggering signal in a flow-based study. The ischemia model outlined above (13) provides a particularly good example by identifying IL-8 in granulocyte arrest. PAF likewise has been convincingly shown to contribute to triggering arrest: a PAF inhibitor decreases arrest without influencing rolling in both a cat ischemia reperfusion model (62) and superoxide-induced vessel injury (63). Models whose readout is recruitment into tissue, although suggestive, cannot distinguish triggering from effects on transmigration. For example, demonstration that anti-IL-8 mAbs inhibit granulocyte influx in rabbit endotoxin-induced pleurisy (64) reveals a critical role for IL-8 in the process as a whole, but does not establish it as a trigger for granulocyte arrest.

A second compelling kind of evidence comes from use of pertussis toxin to inhibit the function of the G-proteins on which the leukocyte serpentine receptors depend for much of their downstream signaling events. Extending earlier observations on pertussis toxin inhibition of lymphocyte homing (65), Bargatze et al (66) demonstrated that it inhibits lymphocyte arrest, but not rolling. Thus, a G-protein–mediated step must precede arrest.

The third line of evidence is that addition of exogenous triggering agents enhances arrest. For example, studies with PAF and LTB4 in vivo demonstrate augmentation of neutrophil adherence and transmigration (7). In vitro, the addition of known triggering agents also increases the frequency of arrest.

The agents used, such as manganese or an integrin-activating antibody, bypass serpentine receptors and act directly on the integrin. Studies in the highly controlled system of lymphocytes that use $\alpha 4\beta 1$ or $\alpha 4\beta 7$ for both primary and secondary adhesion show that such exogenous activation dramatically augments arrest, with minimal effect on rolling (17, 21, 24, 67). Are there circumstances in which acute triggering is not required? Yes—arrest without acute triggering may occur for cells in circulation whose integrins are in the active state, rather than the normal inactive state. This may explain why cells cultured in vitro that are infused back into the host show atypical homing patterns. For example, cultured tumor-infiltrating lymphocytes or IL-2–activated lymphocytes have integrins in an active confirmation and bind to endothelium in vitro without exogenous triggers (68, 69); although some cells do migrate into tumor when infused, the overall pattern is clearly disregulated (69, 70). Similarly, animal models of disease that involve infusion of cultured T cells (such as EAE) may include inappropriate access of cells to tissue compartments due to elimination of the need for a triggering step (and other alternations in their adhesion phenotype). In some experimental model systems, leukocyte arrest occurs with little or no rolling (20, 71). This may reflect either prior triggering or very efficient triggering on contact.

RETENTION OF CHEMOKINE AT THE ENDOTHELIAL SURFACE In order for soluble triggering factors to convey information rapidly during leukocyte-endothelial interactions, they must achieve a threshold local concentration. Most will be rapidly cleared by blood flow (72, 73), although theory predicts limited accumulation in a "concentration boundary layer" close to the vessel wall (74), which might be enhanced by endothelial glycocalyx (75). To circumvent this washout, several strategies may be used by ECs to concentrate factors at or near the endothelial surface. First, the heparin-binding capacity shared by all chemokines (76) may specifically retain them on the proteoglycan-rich glycocalyx of ECs (72). Second, recent IL-8 studies of Middleton et al (77) suggest that chemokine may be concentrated on endothelial protrusions, either in vesicles or at the plasma membrane. Third, the Duffy antigen receptor for chemokines (DARC) is strongly and very selectively expressed on postcapillary venules, the site of virtually all leukocyte emigration (78). DARC is a "promiscuous" chemokine receptor that binds many but not all chemokines; no signaling function has yet been demonstrated. Its functional importance on postcapillary venules has not been proven, but it has the potential to hold chemokines at the endothelial surface and thereby regulate their presentation to leukocytes (either positively or negatively). Furthermore, since transfection studies indicate that DARC can be endocytosed (79), it could play a role in transendothelial transport (described below).

CHEMOKINE RECEPTORS Functional chemokine receptors are selectively expressed on subsets of leukocytes (50). Accordingly, each chemokine acts on a restricted subset of cell types to induce chemotaxis, activation, or adhesion (46–48). Undoubtedly additional chemokine receptors will be identified, adding to the complexity of, and thereby the potential for, information throughput via these receptors. For example, the recently described BLR1-chemokine family receptor is expressed not only on B cells, but also on small specific subsets of CD4 and CD8 T cells (80). Thus, T cells may be as heterogeneous for chemokine receptor expression (and responsiveness) as they are for adhesion molecule expression (2). Both increase the combinatorial sorting capacity of the adhesion cascade.

Triggering during the adhesion cascade must occur on a second or subsecond time scale (66, 71). Although this is extremely rapid compared to most immunologic responses, this is routine for sensory responses. The serpentine receptor family is used for time-critical sensory functions, such as sight and neural transmission (50). When a chemokine binds to its serpentine receptor, it typically generates a calcium flux and phosphoinositide hydrolysis via G-protein coupling (50). The subsequent events must likewise be rapid but are not well understood. We suspect a major role for cytoskeleton given the importance of cytoskeleton to regulating integrin-mediated adhesion (15, 81), the sensitivity of cytoskeleton to calcium and phospholipid mediators (82), and the extreme rapidity of many cytoskeletal events (83, 84).

Issues in Secondary Adhesion

The concepts of secondary adhesion have remained relatively stable over the last several years, with ICAM-1, ICAM-2, and VCAM-1 as the predominant endothelial ligands for strong adhesion. A major solidification has come from the cloning of MAdCAM and demonstration that it is the primary ligand for $\alpha 4\beta 7$ (3, 6). This now substantially explains in molecular terms the preferential lymphocyte migration to the gut. One provocative feature of MAdCAM is its incorporation of both an integrin-ligand and a mucin-ligand for L-selectin (26) in a single molecule. Although it seems not to be a common strategy in the adhesion cascade, it is conceptually a very parsimonious one.

However, surprises probably await. The alternatively spliced form of fibronectin (CS-1) is found on the lumenal aspect of vessels in rheumatoid joints and is a dominant ligand for VLA-4 in vitro (85). Neutrophils can bind to endothelium via a bridge of fibrinogen which in turn is bound to ICAM-1 (86). Likewise, pro-thymocytes show VLA-6-dependent homing to the thymus, which appears, surprisingly, to involve the VLA-6 integrin on the endothelial side of the interaction (10).

ENDOTHELIUM "REPORTS" INFORMATION FROM ITS ENVIRONMENT

Virtually all the information accessible to the passing leukocyte is managed by the EC. There is a fundamental dichotomy between two kinds of information. In one case, for soluble factors such as MIP-1β or IL-8 from other cells, the endothelium can facilitate access of the tissue-derived factor to the lumenal surface, perhaps retaining it there; we refer to this as "posting" (as occurs with endothelial retention described above). Alternatively, the endothelium can integrate incoming signals and "relay" that information by creating a new set of signals for the leukocyte, for example, by regulating synthesis of VCAM-1, E-selectin, or IL-8.

Regulation of expression of adhesion molecules on endothelium has been frequently reviewed (3, 4, 6, 10, 11). Based on the foregoing discussion, we believe that chemokine production by ECs is likewise an important element in the adhesion cascade, and therefore we review the principles and specifics of endothelial production of chemokines. Marked similarities between the rules governing expression of chemokines and adhesion molecules suggest that their regulation has coevolved to optimize the binding of leukocytes.

Chemokine Production by Endothelium

Among the more than 25 chemokines described, at least five of these are expressed by ECs in vivo. MCP-1 and IL-8 appear to be the most "promiscuous" EC-expressed chemokines and have been described extensively in both models, in vivo and in vitro (46, 49). Both chemokines are constitutively expressed in cultured endothelium (87, 88) as well as in apparently normal endothelium in certain tissues in vivo (89–91). Failure to detect IL-8 message in normal heart and brain endothelium may reflect tissue-specific differences in IL-8 transcription in resting endothelium, or simply differences in assay sensitivity (92, 93). It is no more surprising that IL-8 and MCP-1 are transcribed at a basal level in some tissue endothelium, than that ICAM-1 is as well. Their low basal levels may contribute to low frequency T cell binding during recirculation and/or may facilitate rapid induction in inflammatory conditions. They are strongly upregulated by various stimuli [(46), and see below].

In addition to IL-8 and MCP-1, two chemokines have been demonstrated to be produced by endothelium in vivo (IP-10 and RANTES), and three are strong candidates (gro-α/MGSA, Mig, and ENA-78). IP-10 mRNA expression is induced in liver and kidney after intravenous administration of IFN-γ, particularly in the microvasculature (94). RANTES was found to be expressed in ECs surrounding granulomas during DTH reactions in lymph nodes (95).

Gro-α expression has been described in cultured endothelium where it can be induced by a variety of stimuli (96–98). Mig was found recently to be expressed in cultured EC where its regulation was most similar to that of IP-10 (88). Finally, ENA-78 was found at the surface of ECs in synovial tissue (99); it should be noted that finding chemokines localized around endothelium does not necessarily indicate production of the chemokine by that endothelium (100, and see below). Taken together, these findings indicate that ECs can be induced to express members of both the CC- (RANTES, MCP-1) and the CXC-subfamilies (IL-8, Gro-α, IP-10, Mig) of chemokines.

Chemokine Inducers

Many of the agents that induce chemokine expression in EC also induce adhesion molecules relevant to the adhesion cascade. This is consistent with a concept of coordinated display of all required elements for information transfer to the leukocyte. Among the strongest stimuli are the classical inflammatory cytokines TNF-α, IL-1, and IFN-γ (46). Many other soluble mediators can do so also. For example, growth factors such as monocyte-colony-stimulating factor (101) and immune-derived cytokines such as IL-4 and IL-10 (102, 103) induce MCP-1 expression. Likewise, histamine is a potent inducer of IL-8 (87), consistent with a critical role for mast cells (see below). Finally, thrombin can very rapidly induce MCP-1 expression (104), linking chemokine induction to the coagulation cascade.

ECs produce chemokines in response to other important classes of stimuli that are rather different in character from the host protein mediators described above. Microbial products, ischemia, and shear influence chemokine as well as adhesion molecule expression by endothelium. Endotoxin (LPS) from many Gram-negative bacteria is a strong stimulus for chemokine production (46). In addition, capsular polysaccharide from the Gram-positive *Staphylococcus aureus* and outer surface proteins from the spirochete *Borrelia burgdorferi* act as inducers as well (105; and K Ebnet, MM Simon, S Shaw, manuscript in preparation).

Ischemia acts as a potent inducer of MCP-1 and IL-8 production by EC, just as it induces adhesion ligands (92, 93, 106, 107). Such activation of the adhesion cascade by ischemia presumably evolved to deal with wounds but often creates damage in ischemia-reperfusion injuries such as myocardial infarction (108). Finally, mechanical forces such as shear stress generated by blood flow induce MCP-1 mRNA expression in ECs (109), and shear stress–responsive *cis*-acting elements have been identified in the promoters of shear-regulated genes in EC (110). The local expression of chemokines induced by turbulent flow in the vascular tree might therefore contribute to the generation of atherosclerotic lesions (108).

Selectivity in Chemokine Production

There is redundancy of chemokine production by ECs in two respects: Multiple agents induce the same chemokine (e.g. IL-1, LPS, and ischemia all induce IL-8); and a single agent induces multiple chemokines (e.g. TNF induces IL-8, MCP-1, RANTES, and others). However, this does not mean absence of specificity; redundancy in information systems serves to maximize error-free information transmission, and it is often characteristic of biologic cytokine networks (111). Superimposed on this redundancy are elements of specificity, some of which are obvious and some more subtle. The most obvious example of specificity is that endothelium produces only a limited subset of the known chemokines; for example, we know of no evidence for MIP-1α or MIP-1β production by endothelium. Another clear example, as noted above, is that upon intravenous administration of IFN-γ in mice, there is striking preferential induction of IP-10 message in liver and kidney microvascular endothelium; expression on endothelium at other sites is weak or non-existent (94). Thus, in the intact organism, chemokine production by endothelium can be highly specific.

For the most part we do not understand the tissue-derived regulatory elements that confer this kind of specificity in vivo. However, studies in vitro provide some clues. First, we find partial selectivity when looking systematically at patterns of induction of chemokines on cultured endothelium by classic inflammatory mediators. For example, IP-10 and Mig are better induced than MCP-1 by high doses of IFN-γ, while the converse is true for induction by IL-1 (88). Second, additivity/synergy is often seen with the proinflammatory cytokines; endothelial cultures give strong chemokine responses to mixes of TNF-α, IL-1β, and IFN-γ, which when given individually induce minimal response (88, 112). Third, responses to the strong primary inducers such as TNF-α, IL-1β, IFN-γ, or LPS are regulated by other soluble factors. IL-4 and IL-13 partially inhibit the TNF-α/IFN-γ–induced RANTES expression in human umbilical vein endothelial cells (HUVEC) (112). IL-4, however, has also been described as amplifying the IL-1- or LPS-induced production of MCP-1 or IL-8 in HUVEC (113), indicating that the same cytokine can have opposite effects on different chemokines. IL-3 acts cooperatively with TNF-α in the induction of IL-8 and E-selectin expression (114). Finally histamine potentiates IL-8 production induced by TNF-α (87), suggesting that mast cells enhance leukocyte infiltration via chemokine induction.

"Relay" chemokine production by the endothelium itself has theoretical advantages over "posting" of chemokines made by other cells, notably amplification and integration. The foregoing paragraph illustrates the principle of integration of multiple signals. Amplification can be a powerful feature of the relay when the inducing factors are present at low concentration (e.g. early

in the response). For example, less than 1U/ml of TNF-α, IL-1β, and IFN-γ is sufficient to induce more than half-maximal response of IL-8 in cultured endothelium; the lag time in this process is quite modest, since peak levels of IL-8 message are achieved within an hour (88).

HOW INFORMATION REACHES THE ENDOTHELIUM

Short-Range vs Long-Range Information Transfer

Whether the endothelium uses "posting" or "relay" mechanisms to display proadhesive molecules on its lumenal surface, there remains the fundamental problem of how the signal is transported from its source to the vessel. Passive diffusion is of primary value in situations where vessels are in extremely close proximity to the source of the activating signal. Postcapillary venules are within a few cell diameters of various tissue cells including pericytes, stromal cells, and mast cells. Rapid intercellular communication between these cells is illustrated by mast cells; trauma-induced degranulation causes release of small, highly diffusible mediators, including histamine and PAF, that quickly and dramatically initiate endothelial changes (115). Furthermore, inflammatory cells often accumulate in the perivascular region; cytokines generated within these infiltrates have relatively easy access to the adjacent endothelium. Indeed, the extreme example of short-range signaling is that leukocyte membrane-bound IL-1 can induce endothelial IL-8 production via juxtacrine mechanism (116).

In other situations, the signal may need to traverse substantial distances to reach its target vessel. It apparently can do so rapidly: Subcutaneous injection of IL-8 in rats causes a detectable increase in transendothelial migration of lymphocytes in draining lymph nodes within three minutes, peaking at three times normal levels in half an hour (117). This implies extremely rapid transport to lymph node vessels via multiple steps: from inoculation site to dermal lymphatics, to lymph node subcapsular sinus, to paracortical postcapillary venules (see below). Omnidirectional passive diffusion of soluble factors is dissatisfying as an explanation for long-range information transport on several grounds: It is slow, it dilutes the concentration signal exponentially in proportion to the radius of diffusion, and it may be impeded by physical barriers and biochemical interactions within tissues (118). The rules for movement of molecules through tissue are complex and only partially understood (119, and references therein). But as an example, passive diffusion of the growth factor activin appears to occur an estimated speed of around 55 μm per hour (120). In simplified model systems in which a factor such as bFGF is migrating through agarose (with which it has charge interactions), diffusion occurs at about 70 μm per hour. Carrier molecules may increase the speed and radius of diffusion: bFGF bound

to heparin diffuses roughly four times more rapidly through agarose, about 3 mm in 10 h (121). Even so, the speed of passive diffusion is insufficient to account for the rapidity of the lymphocyte recruitment into lymph node described above. Rather, there is evidence for anatomic structures that transport material through tissues and to vessels (122). Of special importance is the concept of fiber bundles in facilitating such transport, which will be illustrated in the lymph node and extended to other tissues.

The Lymph Node Reticular Network

Several studies indicate that soluble tracers arriving via afferent lymphatics penetrate the lymph node parenchyma in a defined and organized pattern (illustrated in Figure 1*A*). The peroxidase tracer arrives at the subcapsular sinus (S), from where it would be expected to diffuse into the upper cortex (C), producing a smooth concentration gradient. Instead, one observes a linear, web-like pattern of accumulation (F) (123). There is prominent accumulation of tracer around and even in the lumen of the high endothelial venule (HEV). This is of particular importance because HEV are the postcapillary venules specialized for the high volume recirculation of lymphocytes into the node. The formation of this pattern reflects a rapid transport mechanism since the micrograph shows tissue fixed 1 min after the tracer entered the sinus. These results were confirmed by studies of Sainte-Marie et al, using FITC-BSA injected into skin of rats (124). Although the route of administration was slightly different—the tracer had to reach lymphatics in the skin before entering the afferent lymphatic—the pattern and sequence of lymph node staining was identical: Tracer entered the subcapsular sinus, streaked downward into the cortex, and reached the HEV. These staining patterns correspond anatomically to the reticular network of the lymph node.

The reticular network has long been thought to be primarily a structural component of the lymph node. It is a network of collagen fibers that extends from the subcapsular sinus region into the deeper cortex and medullary regions. An unusual feature of these fibers is that they are ensheathed throughout most of their course by fibroblastic reticular cells (FRC, Figure 1*B*) (125, 126). As a result, the fibers comprise an extracellular space, effectively insulated from the surrounding lymph node parenchyma that contains lymphocytes and antigen presenting cells. Based on the unique ultrastructural anatomy of the reticular network, Moe proposed in 1963, that "it seems reasonable to hypothesize that the entire reticular interstitium may provide pathways for movement . . . for secretory products" (126). Many of these fibers end by insertion onto the outer surface of the HEV, forming a lace-like sleeve around the vessel (127). Thus, the reticular network is anatomically positioned for the transport of soluble information from the afferent lymph to the HEV. In support of this concept,

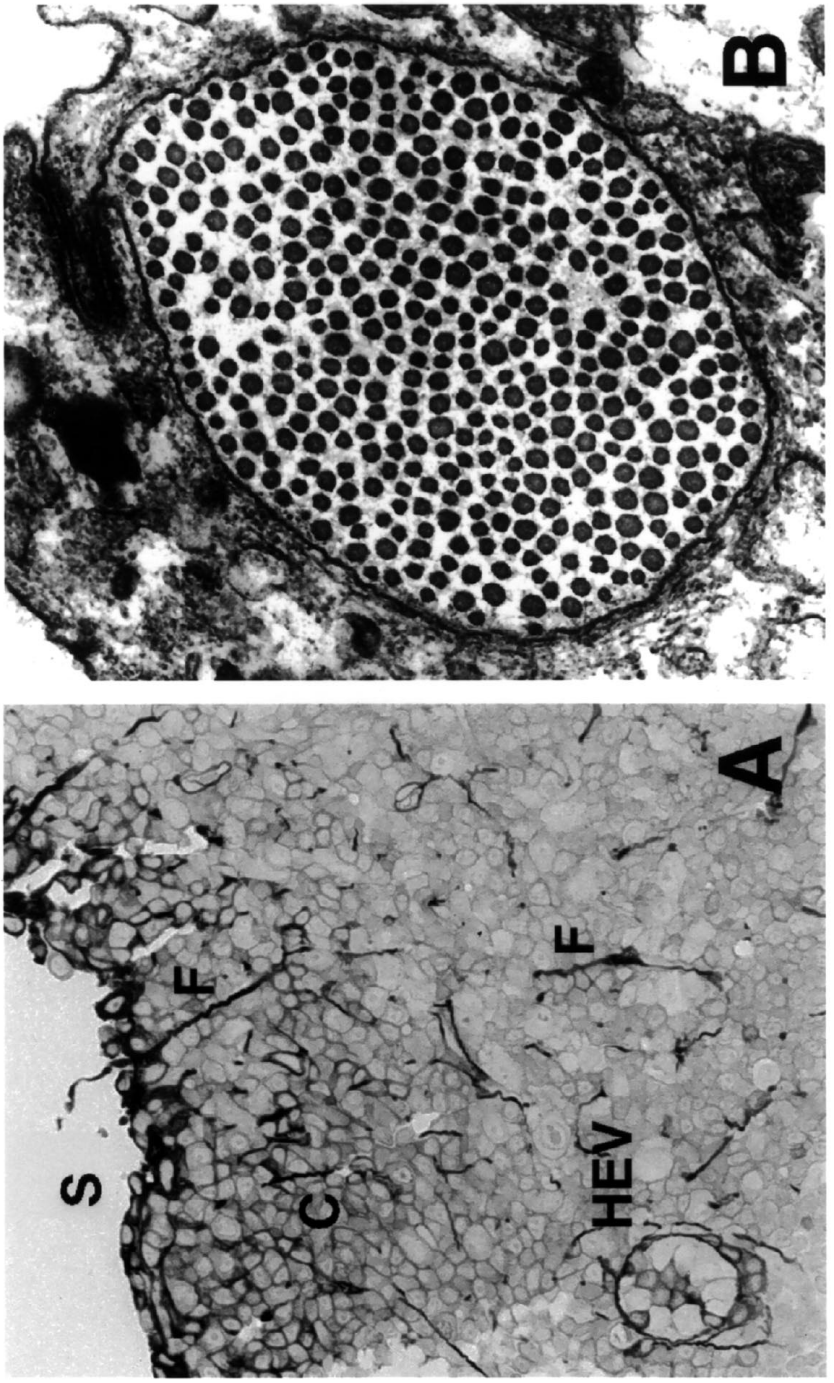

Figure 1 Reticular network of the rat lymph node. *A*. Horseradish peroxidase tracer movement with the reticular network. Tracer was injected into afferent lymphatic. Floor of subcapsular sinus (S), reticular fibers (F), and outer sheath of high endothelial venule (HEV) are stained. Cortex (C) containing lymphocytes is minimally stained. *B*. Sheathing of fiber. Electron micrograph of reticular fiber cross-section showing collagen fibrils enclosed by cytoplasmic processes that are joined at the top of the figure by a junctional complex. Adapted from (75, 123).

immunohistochemical studies of reactive lymph node have localized MIP-1β to the reticular network (EP Kaldjian, ES Jaffe, S Shaw, manuscript in preparation). Although details of the transport mechanism are incomplete, three previously discussed features may contribute (75): solute transport into the reticular network via pinocytosis of lymph by sinus-lining cells; bulk fluid flow along the reticular network facilitated by fluid from fenestrated capillaries within the network; directionality of flow determined by enclosure of the reticular network by its sheath at all but defined locations, particularly HEV.

Fibers as Low-Resistance Pathways in Other Tissues

Fibers also appear to play a role in information transport in other tissues as well. In skin, the anatomic arrangement of elastic fibers and limited experimental data support this concept. The dermal lymphatics are associated with elastic fibers, which radiate perpendicularly from the lymphatic into the surrounding dermis. These fibers are continuous with a network of finer fibers that extend into the upper dermis and between keratinocytes into the epidermis (128). Colloidal carbon particles injected into the dermis localize preferentially to the elastic fibers as they migrate toward initial lymphatics (118, and references therein). Transport along fibers has also been observed in the rabbit mesentery, where low molecular weight fluorescent dyes injected into the mesenteric terminal vascular system move relatively rapidly out from the vessel and along connective tissue fibers in a pattern similar to the reticular pattern of elastic fibers within the tissue (122). Consequently, elastic fibers have been termed a “low resistance” pathway for fluid transport, in contrast to the much slower seepage through surrounding matrix. Sheathed reticular fibers can be found in other lymphoid organs, including spleen, tonsil, and Peyer’s patch of the gut (129). Unsheathed fibers are present in many tissues, including liver sinusoids, neural adventitia, smooth muscle, and tendon. Their role in transport has not yet been evaluated.

Issues in Transendothelial Transport

How do soluble signals such as chemokines gain access to the lumenal surface after movement through surrounding tissue? There are two possibilities and evidence for both. The first is endothelial transport of factors from the abluminal to the lumenal side. Specific uptake and subsequent release of fMLP from cultured ECs suggest this could be a mechanism for its transport (130). Intracutaneous injection of IL-8 results in appearance of large endothelial vesicles whose morphology and kinetics suggested IL-8 transport into the lumen (131). Recently, Rot and colleagues have used immunoelectron microscopy and electron microscopic autoradiography to directly identify IL-8 within such vesicles (77). Time course studies demonstrated progressive accumulation of IL-8 at the lumenal surface, with enrichment on the tips of endothelial protrusions induced

by the IL-8 administration. Thus, endothelial transport of triggering factors to the lumen via an endocytotic mechanism is strongly suggested. Alternatively, soluble factors can move between ECs and into the lumen (36), at least in HEV as shown in Figure 1*A*. Maintenance of the integrity of the vascular permeability barrier is accomplished by the overlapping of adjacent ECs that effectively form a flap valve (75). Regulated movement of fluid and cells across this specialized region is possible because the adjoining endothelial surfaces are held in apposition by discontinuous "spot welds" rather than the occluding junctions characteristic of most endothelia.

CONCLUDING REMARKS

Coordinated flow of information is pivotal to efficient and specific interactions of circulating cells with specialized endothelium: the transport of signals along fiber bundles to vessels, the relay or posting of signals at the endothelial surface, and the ultimate reading of the lumenal display by the leukocyte. The adhesion cascade model has transformed our understanding of the binding itself. Much remains to be elucidated regarding the flow of information that dictates the specificity of that binding.

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THE INTERLEUKIN-2 RECEPTOR γ CHAIN: Its Role in the Multiple Cytokine Receptor Complexes and T Cell Development in XSCID

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KEY WORDS: interleukin 2, cytokine receptor, T cell development, XSCID, signal transduction

ABSTRACT

Interleukin 2 (IL-2), a T cell–derived cytokine, targets a variety of cells to induce their growth, differentiation, and functional activation. IL-2 inserts signals into the cells through IL-2 receptors expressed on cell surfaces to induce such actions. In humans, the functional IL-2 receptor consists of the subunit complexes of the α, β, and γ chains, or the β and γ chains. The third component, the γ chain, of IL-2 receptor plays a pivotal role in formation of the full-fledged IL-2 receptor; together with the β chain, the γ chain participates in increasing the IL-2 binding affinity and intracellular signal transduction. Moreover, the cytokine receptors for at least IL-2, IL-4, IL-7, IL-9, and IL-15 utilize the same γ chain as an essential subunit. Interestingly, mutations of the γ chain gene cause human X-linked severe combined immunodeficiency (XSCID) characterized by a complete or profound T cell defect. Among the cytokines sharing the γ chain, at least IL-7 is essentially involved in early T cell development in the mouse organ culture system. The molecular identification of the γ chain brought a grasp of the structures and functions of the cytokine receptor and an in-depth understanding of the cause of human XSCID. To investigate the mechanism of XSCID and development of

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gene therapy for XSCID, knockout mice for the γ chain gene were produced that showed similar but not exactly the same phenotypes as human XSCID.

INTRODUCTION

A variety of cytokines have been molecularly identified as soluble factors regulating the immune system, hematopoietic system, and other cell-cell interactions. They exert pleiotropic and redundant functions via their receptors expressed on multiple target cells (1). For understanding such cytokine actions, a series of cytokine receptors have also been molecularly identified and functionally characterized. Cytokine receptors are classified into at least five distinct families based on the structural characteristics of their extracellular and intracellular domains: the cytokine receptor superfamily, interferon receptor family, TNF receptor family, TGF-β receptor family, and IL-8 receptor family (2). The cytokine receptor superfamily is the largest family, containing at least 18 distinct receptor molecules, some of which may be shared among multiple cytokine receptors; the receptors for IL-3, IL-5, and GM-CSF contain the common β chain, and the receptors for IL-6, IL-11, OSM, CNTF, and LIF contain the common gp130. In essence, the common β chain and gp130 participate in increasing ligand-binding affinities and in ligand-induced intracellular signal transduction, suggesting that the sharing of receptor subunits may in part reflect the redundant functions of cytokines (2, 3).

The IL-2 receptors were originally classified into three isoforms, the high-, intermediate-, and low-affinity IL-2 receptors (4). Molecular characterization of the IL-2 receptor commenced with gene cloning of the α chain (IL-2Rα), which is unnecessary for intracellular signal transduction mediated by IL-2 (5–7). The second subunit of IL-2 receptor, β chain (IL-2Rβ), was then characterized as belonging to the cytokine receptor superfamily and as essential for the intracellular signal transduction (8). Introduction of IL-2Rα and IL-2Rβ genes induced the functional high-affinity IL-2 receptor in lymphoid cells but not in fibroblastoid cells, suggesting the possible existence of the lymphoid specific third component, the γ chain (IL-2Rγ), of the IL-2 receptor. Molecular identification of IL-2Rγ was achieved by coimmunopurification with IL-2Rβ, demonstrating that IL-2Rγ has the structural profile specific for the cytokine receptor superfamily, as does IL-2Rβ (9). Not only IL-2Rβ but also IL-2Rγ is essentially involved in the intracellular signal transduction, although their cytoplasmic domains do not contain any known effector function for signal transduction. However, associations of several effector molecules with their cytoplasmic domains have been revealed; in particular, novel tyrosine kinases Jak1 and Jak3 are physically bound with the serine-rich

region of IL-2Rβ and the region containing the SH2 subdomains of IL-2Rγ, respectively (10).

Similar to the common β chain and gp130, IL-2Rγ was found to be shared with multiple cytokine receptors other than IL-2 receptor, such as receptors for IL-4, IL-7, IL-9, and IL-15 (10). Furthermore, the sharing of IL-2Rβ between IL-2 receptor and IL-15 receptor was recently found (11). All such receptor subunits shared by multiple cytokine receptors essentially participate in increasing ligand binding affinities and in ligand-induced intracellular signal transduction, suggesting that the sharing of receptor subunits may in part reflect the redundant functions of cytokines. The redundancy of cytokines may result in prevention of severe immunodeficiencies in mice targeted for cytokine genes; for example, gene-targeted mice for IL-2 and IL-4, both of which are capable of promoting T cell proliferation, carried normal numbers of T cells (12–14). However, knockout mice for IL-2Rβ were recently reported to show the deregulated T cell activation that resulted in autoimmunity (15). On the other hand, dysfunction of IL-2Rγ causes human XSCID, characterized by a profound defect of T cells (16). Consequently, the cytokines sharing IL-2Rγ should be implicated in the early T cell development.

THE IL-2/IL-2 RECEPTOR SYSTEM

Molecular Characterization of Receptor Subunits

At least three distinct subunits—IL-2Rα, IL-2Rβ, and IL-2Rγ—constitute IL-2 receptor complexes. The schematic structures of the human subunits are shown in Figure 1. The human IL-2Rα was originally detected using a monoclonal antibody (mAb) recognizing the Tac antigen expressed on activated T cells and leukemic cell lines carrying HTLV-I, and then it was molecularly cloned (5–7, 17). The human IL-2Rα gene is organized into eight exons spanning more than 35 kb and localized on chromosome 10p l4–15 (18, 19). The mature form of IL-2Rα, deduced from the nucleotide sequence, consists of 251 amino acid residues with no significant homology to known cytokine receptors except the recently identified α chain of IL-15 receptor (20) (Figure 1). The cytoplasmic domain of the IL-2Rα contains only 13 amino acid residues, which seems insufficient to harbor a signal transducing capacity. The second subunit β chain of IL-2 receptor (IL-2Rβ) was initially identified to be a 75-kDa cell surface glycoprotein, by affinity cross-linking experiments with radiolabeled IL-2 (21–25) and subsequently by mAbs specific for the human IL-2Rβ (26, 27). The complete cDNA clone encoding the human IL-2Rβ was isolated by expression cloning with the mAbs (8). The human genomic IL-2Rβ gene is partitioned into 10 exons, spanning 24 kb on chromosome 22q11.2–12 (28, 29). The

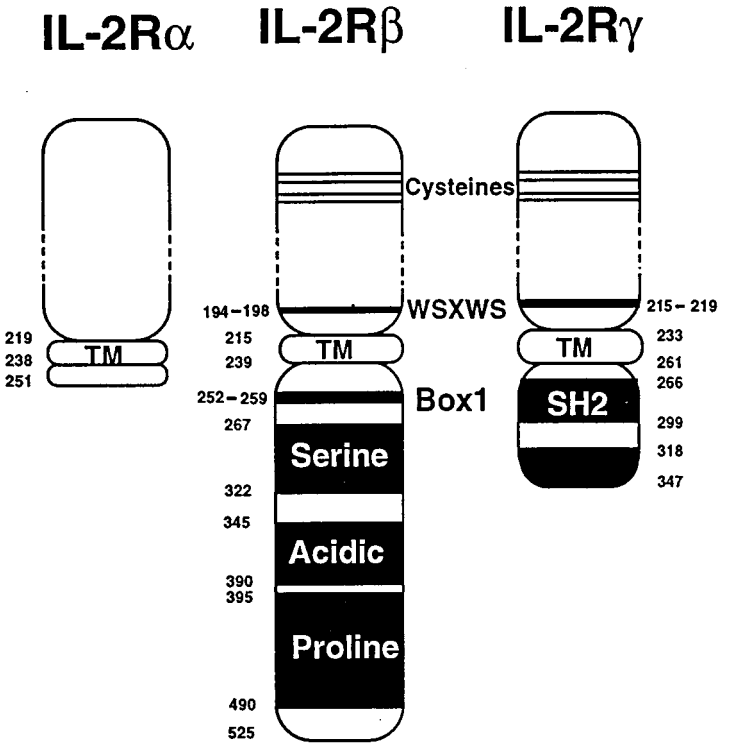

Figure 1 The schematic structure of the IL-2 receptor subunits, IL-2Rβ and IL-2Rγ. The numbers are amino acid positions from the amino terminals. The solid lines indicate the conserved cysteine residues. WSXWS indicates an amino acid sequence of tryptophan—serine—unconserved amino acid—tryptophan—serine. Box 1, Serine, Acidic, and Proline in the cytoplasmic domain of IL-2Rβ represent the box 1, serine-rich, acidic, and proline-rich regions, respectively. SH2 and C30 in the cytoplasmic domain of IL-2Rγ represent the SH2 subdomains and carboxyl terminal 30 amino acid residues, respectively.

mature form of IL-2Rβ consists of 525 amino acid residues. Characterized as a member of the cytokine receptor superfamily, IL-2Rβ has the common features of two pairs of the conserved cysteine residues near the amino-terminal and a sequence of tryptophan—serine—x(unconserved amino acid)—tryptophan—serine (WSXWS, WS motif) in the extracellular domain (30). The cytoplasmic domain of IL-2Rβ, consisting of 286 amino acid residues, contains unique regions such as the box 1, serine-rich, acidic, and proline-rich regions. The third subunit γ chain of IL-2 receptor (IL-2Rγ) was first detected by coimmunoprecipitation with IL-2Rβ prior to the IL-2Rβ gene cloning (31, 32).

TU11 mAb specific for the human IL-2Rβ precipitated a 64-kDa cell surface molecule distinct from IL-2Rα, together with IL-2Rβ in lysates of IL-2-treated cells expressing the high-affinity IL-2 receptor (31). The numbers of IL-2Rβ molecules on lymphoid transfectants with the IL-2Rβ gene usually exceeded sites of the intermediate-affinity receptor, which was known to contain IL-2Rβ but not IL-2Rα. The amount of the 64-kDa molecule coprecipitated with IL-2Rβ correlated well with the level of the intermediate-affinity IL-2 binding sites, suggesting the possibility that the 64-kDa molecule is IL-2Rγ (32). The 64-kDa molecule was purified by coimmunoprecipitation with IL-2Rβ, and its amino-terminal amino acid residues were determined. Based on the amino acid sequence, the complete cDNA clone encoding the 64-kDa molecule was isolated and demonstrated to be the cognate IL-2Rγ chain (9). The mature form of IL-2Rγ consists of 347 amino acid residues with sequences typical of the cytokine receptor superfamily such as IL-2Rβ. The cytoplasmic domain of IL-2Rγ, consisting of 86 amino acid residues, contains two subdomains of the Src homology region 2 (SH2). The full SH2 domain is known to contribute to the downstream signaling through its interaction with phosphotyrosine residues of various signal transducing effector molecules, but the two SH2 subdomains detected in IL-2Rγ are thought to be insufficient for such action (33). As described later, IL-2Rγ participates in formation of functional cytokine receptors not only for IL-2 but also for IL-4, IL-7, IL-9, and IL-15, and its dysfunction results in the occurrence of human XSCID.

Reconstitution of IL-2 Receptor Complexes

Expression of IL-2 receptor has been detected on hematopoietic cells and glioma cell lines but not on other nonhematopoietic cells including fibroblastoid cells and epithelial cells. Therefore, to investigate functional significances of the three distinct IL-2 receptor subunits, expression plasmids for human IL-2Rα, IL-2Rβ, or IL-2Rγ gene were stably transfected into the L929 fibroblastoid cell line, and the transfectant clones expressing various combinations of the receptor subunits were established. They were examined for their association, dissociation rate constants and affinities for IL-2 binding (9, 34) (Figure 2). Expression of IL-2Rα alone or of both IL-2Rα and IL-2Rγ showed low affinities (Kd $= 10^{-8}$) to IL-2 binding, and either IL-2Rβ or IL-2Rγ alone possessed undetectable affinities (Kd $> 10^{-7}$ M) for IL-2 binding. The association rate constant with the $\alpha\beta\gamma$ heterotrimer complex was fourfold larger than that with the $\alpha\beta$ heterodimer complex, and the dissociation rate constant was one fifth of that with the $\alpha\beta$ heterodimer complex, resulting in two different types of high-affinity receptors with Kd of 10^{-11} and 10^{-10} M, respectively. Since the $\alpha\beta$ heterodimer complex has no signal transducing ability for cell growth, it is referred to as the pseudo-high-affinity receptor. On the other hand, the $\beta\gamma$

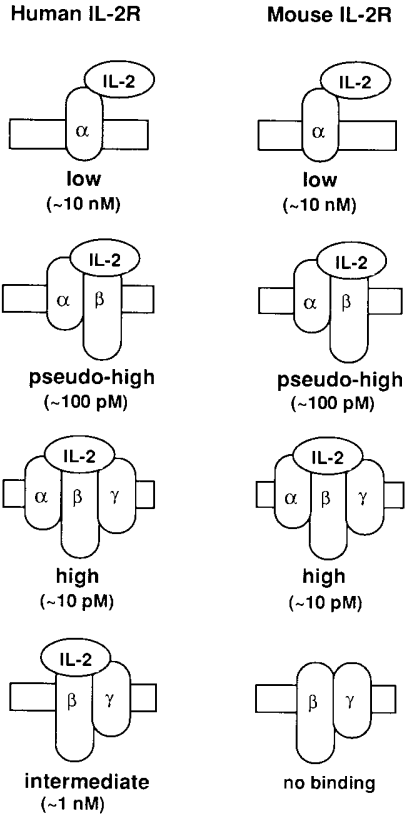

Figure 2 The human and mouse IL-2 receptor complexes and their affinities for IL-2 binding. The numbers in parentheses indicate IL-2 binding affinities.

heterodimer complex exhibited intermediate affinities (Kd $= 10^{-9}$ M). The dissociation rate constants of the $\alpha\beta\gamma$ and $\beta\gamma$ complexes on lymphoid cells were generally much lower than those on fibroblastoid cells, suggesting that there is a significant difference between these two types of cells in regulating the dissociation of IL-2 from the receptors. The α chains of other cytokine receptors, such as IL-3, IL-5, or GM-CSF, and IL-6 or CNTF possess low affinities by themselves, but the heterodimers of the α chains with the β chains of such receptors form high-affinity receptors. These suggest a functional analogy between IL-2Rα and the α chains of other cytokine receptors.

IL-2 receptors reconstituted on fibroblastoid cells were also examined for their ability to transduce IL-2–mediated intracellular signals, as described later

in detail. The transfectants expressing the human $\alpha\beta\gamma$ and $\beta\gamma$ complexes of IL-2 receptor responded to IL-2 in terms of tyrosine phosphorylation of IL-2Rβ and induction of protooncogenes such as *c-myc*, *c-fos* and *c-jun* (35). On the other hand, the transfectants expressing the $\alpha\beta$ complex or the $\alpha\gamma$ complex showed unresponsiveness to IL-2. These indicate that both human IL-2Rβ and IL-2Rγ are essential and adequate for formation of functional IL-2 receptor complexes; IL-2Rα only increases the IL-2 binding affinity of the IL-2 receptor $\beta\gamma$ complex.

Similar reconstitution experiments of IL-2 receptor complexes were performed with expression plasmids for mouse receptor subunit genes, in an effort to characterize the difference between the human and mouse IL-2 receptor systems. The mouse $\alpha\beta\gamma$, $\alpha\beta$, and $\alpha\gamma$ complexes possess association and dissociation rate constants similar to those of human complexes, but the mouse $\beta\gamma$ complex has an undetectable affinity to IL-2 binding (36) (Figure 2). Such a difference in the IL-2 binding affinity of the $\beta\gamma$ complex between human and mouse was seen not only in transfectant cell lines but also in normal lymphocytes. Both human and mouse CD8$^+$ T cells and NK cells express the $\beta\gamma$ complex of IL-2 receptor, as described later. Although human CD8$^+$ T cells and NK cells exhibited IL-2 binding ability and IL-2 responsiveness, at least mouse CD8$^+$ T cells were unresponsive to IL-2 and incapable of binding IL-2 (36). Therefore, the three receptor subunits including IL-2Rα are indispensable for formation of the functional IL-2 receptor in the mouse system.

Expression of IL-2 Receptor Subunits

Expressions of IL-2Rα, IL-2Rβ, and IL-2Rγ on various populations of human peripheral blood cells were examined by staining with mAbs specific for each receptor subunit (37–39). IL-2Rγ expression was seen on all of the populations including CD4$^+$ T, CD8$^+$ T, CD20$^+$ B, CD56$^+$ NK cells, and CD14$^+$ monocytes. The granulocyte population was also positive for IL-2Rγ. On the other hand, IL-2Rα and IL-2Rβ were differentially expressed on these cell populations, although their expressions were enhanced by extracellular stimuli such as antigens and mitogens. CD8$^+$ T cells and CD56$^+$ NK cells significantly expressed IL-2Rβ, but little of IL-2Rα, while CD4$^+$ T cells expressed faint amounts of IL-2Rβ. Such a differential expression of IL-2Rβ on CD4$^+$ T and CD8$^+$ T cells reflects their respective IL-2 responsiveness, because the human $\beta\gamma$ complex forms functional receptor. In fact, CD8$^+$ T and NK cells freshly prepared from the peripheral blood showed a strong proliferative response to IL-2, whereas CD4$^+$ T cells required stimulation with macrophages for their IL-2 responsiveness (40, 41). It is of interest that CD16-CD56$^+$ NK cells in the human early pregnancy decidua express the high-affinity IL-2 receptor

consisting of the $\alpha\beta\gamma$ complex, suggesting that these NK cells may be activated in vivo (42).

Mouse splenic cell populations exhibited an expression pattern similar to that of the three receptor subunits to the human peripheral blood cell populations (43). Furthermore, mouse thymocytes were also examined for expression of the IL-2 receptor subunits and the α chains of IL-4 and IL-7 receptors that share IL-2Rγ as a common receptor subunit. The double negative ($CD4^-/CD8^-$) T cells, which are the most immature subset of T cells in thymus, were significantly positive for IL-2Rγ, IL-2Rα, and IL-7Rα. They were however almost negative for IL-2Rβ and IL-4Rα, predicting that double negative T cells express functional IL-7 receptor but little of functional IL-2 and IL-4 receptors (10). The double positive T cell subset contained a small population of IL-2Rγ positive cells and a large population of IL-4Rα positive cells but was negative for IL-2Rα, IL-2Rβ, and IL-7Rα, predicting that a small population of double positive T cells would express the functional IL-4 receptor, and that most of the double positive T cells would have no functional receptors for IL-2 and IL-7 (10).

IL-2Rγ is constitutively expressed on various populations of human and mouse hematopoietic cells, while expressions of IL-2Rα and IL-2Rβ chains are restricted to lymphocytes and monocytes/macrophages. Furthermore, IL-2Rα and IL-2Rβ expressions are different among cell populations, but they are known to be induced or enhanced within a day after stimulation with mitogens (37, 38). In contrast to IL-2Rα and IL-2Rβ expressions, IL-2Rγ expression on normal activated T cells was significantly suppressed by IL-2 stimulation. The IL-2-induced suppression of IL-2Rγ expression was also demonstrated by IL-2Rγ promoter-driven luciferase assays (44). Since IL-2Rγ is essential for the functional IL-2 receptor, the IL-2-induced suppression of IL-2Rγ expression may result in cessation of IL-2-dependent T cell growth. On the other hand, HTLV-I-infected human T cells are often established as IL-2-dependent cell lines, and such cell lines constitutively express the high-affinity IL-2 receptor (45). In these HTLV-I-infected T cell lines, IL-2 did not induce suppression of IL-2Rγ expression. In fact, a transacting transcriptional activator HTLV-I Tax was found to augment expression of IL-2Rγ; moreover, Tax nullified the IL-2–mediated suppression of IL-2Rγ expression (44).

IL-2Rγ IS A COMMON SUBUNIT FOR MULTIPLE CYTOKINE RECEPTORS

Sharing with the IL-4 Receptor

The cytokine receptor superfamily is known to include the common components for multiple cytokine receptors such as the β chain of receptors for IL-3, IL-5,

and GM-CSF, gp130 of receptors for IL-6, IL-11, OSM, LIF, and CNTF, and the β chain of receptors for LIF, OSM, and CNTF (Figure 3) (2, 3). Expression of IL-2Rγ is detectable in a wide range of hematopoietic cell populations as distinct from IL-2Rα and IL-2Rβ expressions, allowing us to suppose that IL-2Rγ, apart from IL-2, serves as a multireceptor subunit. This supposition was suggested by the findings that human XSCID characterized by a T cell defect is caused by mutations of the IL-2Rγ gene (16), and that IL-2-deficient SCID patients and mice carry the normal phenotype of T cells (12–14, 46–48). IL-2Rγ was then predicted to be a common subunit of receptor complexes for IL-2 and other cytokines that may be necessary for early T cell development. To demonstrate such sharing of IL-2Rγ among multiple cytokine receptors, we and others applied two distinct procedures: one, blocking of cytokine functions by mAbs specific for the mouse IL-2Rγ, and two, reconstitution of cytokine receptors with transfection of IL-2Rγ and other cytokine receptor subunit genes. Candidate cytokines sharing the IL-2Rγ were expected to include cytokines affecting T cells. We established two types of mAbs, TUGm2 and TUGm3, specific for the mouse IL-2Rγ: TUGm2 can block the specific interaction between IL-2 and IL-2Rγ, and TUGm3 can precipitate IL-2Rγ cross-linked with IL-2. Using these mAbs, we have obtained evidence of IL-2Rγ sharing with receptors for IL-4, IL-7, and IL-9.

Initially identified as a B cell growth factor, IL-4 is known to possess the capacity to promote growth of T and mast cells (49). The α chain of IL-4 receptor (IL-4Ra) was identified as a 140-kDa molecule consisting of 800 amino acid residues, a member of the cytokine receptor superfamily (50). IL-4 responsive lymphoid cell lines expressed the high-affinity IL-4 receptor, whereas nonlymphoid COS-7 transfectant with the IL-4Rα gene expressed the IL-4 receptor with a lower affinity than that of the high-affinity IL-4 receptor on hematopoietic cells, suggesting that the high-affinity IL-4 receptor on lymphoid cells consists of a complex composed of at least IL-4Rα and another subunit. Consequently, IL-2Rγ was first examined for its sharing with the IL-4 receptor complex. TUGm2 significantly suppressed IL-4-dependent growth of CTLL-2 cells (51). Furthermore, in IL-4 binding assays, the binding of the high-affinity IL-4 receptor on CTLL-2 cells was significantly reduced by their treatment with TUGm2 (from 130 pM to 370 pM), although the IL-4 binding sites were unchanged (51). Since IL-2Rγ itself has no ability to bind IL-4 directly, IL-2Rγ was expected to form a complex with IL-4Rα. The direct participation of IL-2Rγ in the IL-4 receptor complex was then demonstrated by immunoprecipitation with another mAb specific for IL-2Rγ, TUGm3. CTLL-2 cells were treated with IL-4 and then chemically cross-linked, and their lysates were immunoprecipitated by TUGm3. IL-4-cross-linked IL-2Rγ was seen in the

immunoprecipitate, indicating the physical interaction between IL-4 and IL-2Rγ (51). All these results suggest the sharing of IL-2Rγ with IL-4 receptor (Figure 3). Similar results were obtained from the reconstitution experiments of IL-4 receptors by cotransfection of IL-2Rγ and IL-4Rα genes (52).

On the other hand, differential involvement of IL-2Rγ in formation of functional IL-4 receptors has been suggested; although TUGm2 significantly inhibited IL-4-induced proliferation of mouse BA/F3 cells, IC2 cells, and splenic B cells, it showed no effect on IL-4-induced expression of MHC class II molecules and CD23 on the cells (53). A possible physical interaction between IL-4 receptor and IL-13 has been suggested; IL-13 competitively inhibited binding of IL-4 to the functional IL-4 receptor (54, 55). Although IL-2Rγ is not shared with the functional IL-13 receptor, the sharing of IL-4Rα and an unknown component between receptors for IL-4 and IL-13 has been predicted (56, 57) (Figure 3).

Sharing with the IL-7 Receptor

Initially detected as a growth factor for pre-B cells derived from mouse bone marrow stromal cells, IL-7 was found to induce in vitro proliferation of T cells in combination with TPA stimulation, which proceeded in an IL-2-independent manner (58–60). Furthermore, IL-7-dependent proliferation of double negative thymocytes was seen in thymic organ cultures (61). Thus, the biological significance of IL-7 in B and T cell development was suggested. The α chain of human IL-7 receptor (IL-7Rα) was isolated by direct expression cloning strategy (62). The mature form of IL-7Rα consists of 439 amino acid residues with a calculated molecular weight of 49.5 kDa. The extracellular domain of IL-7Rα contains the features of the cytokine receptor superfamily, and the cytoplasmic domain with 195 amino acids in length does not contain consensus sequences for protein kinases. COS-7 transfectants with IL-7Rα expressed IL-7 receptors, of which the IL-7 binding affinity was significantly lower than that of the high-affinity IL-7 receptor expressed on lymphoid cells. These results suggest that the high-affinity IL-7 receptor on lymphoid cells consists of a complex composed of IL-7Rα and another receptor component. Thereby, IL-2Rγ was suspected to be a common receptor subunit shared with the IL-7 receptor. Although IL-2Rγ expressed on fibroblastoid transfectant cells were incapable of binding IL-7, IL-7-dependent proliferations of a mouse pre-B cell line, IxN/2b, and Con A-stimulated splenic cells were significantly suppressed by treatment with TUGm2, a blocking mAb specific for mouse IL-2Rγ (63). Simultaneous treatment with TUGm2 and A7R34, a mAb specific for mouse IL-7Rα, induced complete inhibition of the IL-7-dependent cell proliferation. The Scatchard analysis for IL-7 binding showed that IxN/2b cells express the high- and low-affinity IL-7 receptors, and their treatment with TUGm2 reduces the affinity of

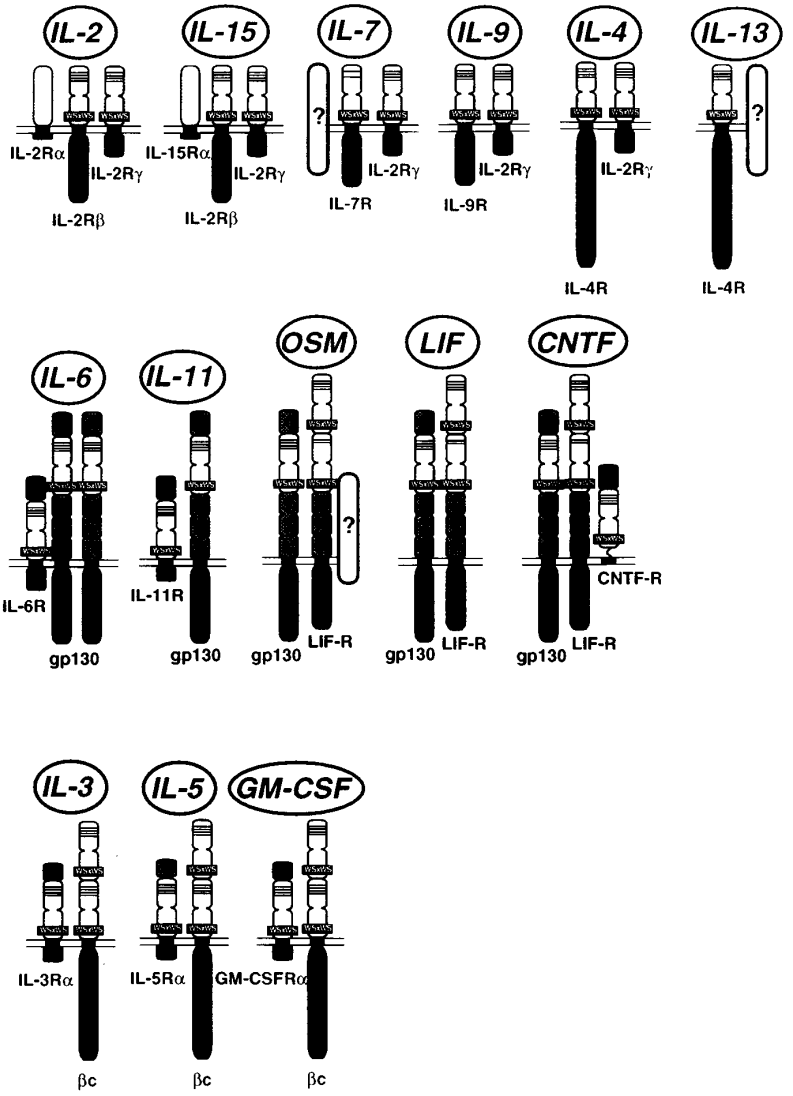

Figure 3 Sharing of the receptor subunits among multiple cytokine receptors.

the high-affinity IL-7 receptor from 79 pM to 255 pM without affecting the low-affinity receptor (63). Treatment of cells with both TUGm2 and A7R34 eliminated the high-affinity receptor, but the low-affinity receptor was unchanged. These results are similar to those of the IL-2/IL-2 receptor system, suggesting the possibility that the high-affinity IL-7 receptor consists of a tripartite complex composed of IL-7Rα, IL-2Rγ, and another unknown component that is expected to be involved in formation of the low-affinity receptor. An IL-7 receptor complex without IL-2Rγ may comprise the intermediate-affinity IL-7 receptor with a Kd of 255 pM. In a way similar to the IL-4 receptor, the physical association of IL-2Rγ with IL-7 was shown in the chemical cross-linking experiments. Radiolabeled IL-7-bound IxN/2b cells were chemically cross-linked and immunoprecipitated by TUGm3 (63). The immunoprecipitates apparently contained IL-2Rγ cross-linked with IL-7 in addition to other molecules, which may include IL-7-cross-linked IL-7Rα. All these observations indicate that IL-2Rγ is shared with the functional high-affinity IL-7 receptor (Figure 3). The reconstitution experiments of IL-7 receptors by cotransfection of IL-2Rγ and IL-7Rα genes also provided evidence of the IL-2Rγ sharing with IL-7 receptor (64).

Sharing with Other Cytokine Receptors

The sharing of IL-2Rγ with multiple cytokine receptors was further examined using IL-9 and IL-15, both of which have the ability to promote T cell proliferation. The α chain of IL-9 receptor (IL-9Rα) belongs to the cytokine receptor superfamily, and it consists of 483 amino acid residues (65). Its cytoplasmic domain contains a region highly homologous to IL-2Rβ, suggesting a functional similarity between IL-9Rα and IL-2Rβ. Since IL-2Rβ was known to form a complex with IL-2Rγ, IL-2Rγ was predicted to participate in formation of an IL-9 receptor complex. To assess this possibility, two mouse IL-9-responsive cell lines, MC/9 and CTLL-2, were examined for the effect of TUGm2 on their proliferation in response to IL-9. Their IL-9-dependent proliferations were almost completely inhibited by their treatment with TUGm2, suggesting the involvement of IL-2Rγ in formation of the functional IL-9 receptor (66). Unlike the other cytokine receptors sharing IL-2Rγ, however, Scatchard analysis showed no effect of TUGm2 on the affinity of IL-9 binding (66). On the other hand, the direct interaction of IL-9 with IL-2Rγ was shown by chemical cross-linking experiments. TUGm3 precipitated IL-9-cross-linked IL-2Rγ in addition to other molecules, which may include IL-9-cross-linked IL-9Rα (66). These observations indicate the sharing of IL-2Rγ with IL-9 receptor (Figure 3); they suggest that IL-2Rγ participates in the IL-9-mediated signal transduction but not in increasing the IL-9 binding affinity of the receptor. Another mAb specific for IL-2Rγ also reportedly induces inhibition of IL-9 responsiveness (67).

IL-15 was originally detected in supernatants of a simian kidney epithelial cell line, CV-1/EBNA, as an IL-2-like T cell growth factor mediating proliferation of IL-2-dependent cells. Recombinant IL-15 was found to induce proliferation of CTLL-2 and PHA-stimulated T cells, which also are responsive to IL-2 (11). The functional similarity between IL-15 and IL-2 was further accentuated by the finding that a mAb specific for IL-2Rβ significantly inhibits the biological activities of IL-15 (11, 68). Therefore, the interaction of IL-15 with the IL-2 receptor was then subjected to thorough investigation. The results revealed the sharing not only of IL-2Rβ but also of IL-2Rγ with the functional IL-15 receptor (69) (Figure 3). Recently, the α chain of IL-15 receptor was analyzed at the molecular level; it possesses a structure homologous to IL-2Rα (20) (Figure 3). The functional IL-15 receptor has been demonstrated to consist of a complex of IL-15Rα, IL-2Rβ, and IL-2Rγ, or a complex of IL-2Rβ and IL-2Rγ.

SIGNALING PATHWAYS FROM THE IL-2 RECEPTOR

Involvement of the Cytoplasmic Domain of IL-2Rβ

Most receptors for growth factors such as CSF-1, PDGF, EGF, and FGF possess intrinsic tyrosine kinase activities, whereas the ligand-induced activation of tyrosine kinases essentially contributes to intracellular signal transductions (70). Both IL-2Rβ and IL-2Rγ are indispensable subunits for the functional IL-2 receptor complex, which participate in increasing the IL-2 binding affinity and intracellular signal transduction (9, 35). The heterodimerization of the cytoplasmic domains of IL-2Rβ and IL-2Rγ generates intracellular signals for cell proliferation in T cells (71, 72), while the homodimerization of IL-2Rβ seems enough for signal transduction for cell proliferation in B cells (73). Both IL-2Rβ and IL-2Rγ belong to the cytokine receptor superfamily, of which the cytoplasmic domains, however, do not contain any consensus motifs of effector molecules for intracellular signal transduction, such as tyrosine kinases. However, they are reportedly associated with several nonreceptor-types of protein tyrosine kinases, whose activations mediated by ligand stimulation are thought to be important for signal transductions (74). The downstream signals mediated by the growth factors involve activations and cascade interactions of various signal transducing effector molecules, such as PI3 kinase, PLCγ, Grb2, SOS, Ras, GAP, Raf-1, MAP, kinase, and so on. These biochemical events are also known to be generated in the signaling pathways from various cytokine receptors including the IL-2Rβ and IL-2Rγ complex (10).

To elucidate the molecular basis for the signal transduction from the IL-2 receptor, various cytoplasmic deletion mutants of the IL-2Rβ and IL-2Rγ were

prepared and used for reconstitution of the receptor complex together with the wild type of each subunit. The mutants of human IL-2Rβ prepared had the serine-rich region, the acidic region, and most of the cytoplasmic domain deleted. The IL-2 receptor reconstitution studies with these IL-2Rβ mutants revealed that the serine-rich region of IL-2Rβ plays a crucial role in the IL-2–mediated signal transduction for cell growth and c-*myc* induction (75, 76). The acidic region of IL-2Rβ is associated with the Src type tyrosine kinases such as Lck and Fyn and probably Lyn (77, 78). The acidic region deletion mutant of IL-2Rβ failed to induce c-fos and c-jun expression in transfectant clones of a mouse IL-3-dependent pro-B cell line, BAF-B03, suggesting possible involvement of the Src-type tyrosine kinases in signaling for c-fos and c-jun induction (79). However, we have recently observed induction of c-fos and c-jun expression in transfectant clones of a mouse T cell line EL-4 with the acidic region deletion mutant of human IL-2Rβ, which (the transfectant clones) express the mutant IL-2Rβ and intrinsic IL-2Rγ (K Oda, H Asao, M Nakamura, and K Sugamura, unpublished observation). Therefore, there seems to be some element of controversy regarding the relationship between the inducing signal for c-fos and c-jun expression and the acidic region of IL-2Rβ. On the other hand, the serine-rich region of IL-2Rβ is associated with two types of tyrosine kinases such as Jak1 and Syk. The significance of Jak1 in signal transduction has not yet been defined, a point described later. Syk was activated in T cells rapidly after IL-2 stimulation, and its activation may have been involved in the c-myc induction (80).

Other effector molecules such as Shc and PI3 kinase were also associated with the cytoplasmic domain of IL-2Rβ (81, 82); in particular, PI3 kinase is reportedly associated with phosphorylated Tyr^{392} of IL-2Rβ (83). Activation of PI3 kinase was regulated by Fyn tyrosine kinase activated by IL-2 in IL-2-responsive T cells (84). Moreover, a recent study suggested that IL-2-induced activation of PI3 kinase leads to activation of the MAP kinase activator MEK in T cells, which is a novel signaling pathway (85). Shc is associated with Sos and acts upstream of Ras. In fact, Ras was previously shown to be activated in T cells stimulated by IL-2, and both the serine-rich and the acidic regions of IL-2Rβ were essentially involved in the activation of Ras (86).

The box 1 and box 2 regions were originally defined in the cytoplasmic domain of gp130 of IL-6 receptor and conserved among several cytokine receptors (87). The box 1 and box 2 of IL-2Rβ are located close to the transmembrane domain and in the serine-rich region, respectively. The replacement of Leu^{299} to Pro in the box 2 of IL-2Rβ rendered the IL-2 receptor incapable of cell growth signal transduction (88), and similarly, the substitution of Ala for ASP^{258} in the box 1 of IL-2Rβ markedly compromised such receptor function (89). These

findings suggest the importance of the box 1 and box 2 regions of IL-2Rβ for receptor functions. The interaction of the box 1 with Jak1 tyrosine kinase is discussed later.

Involvement of the Cytoplasmic Domain of IL-2Rγ

The cytoplasmic deletion mutants of IL-2Rγ prepared had the carboxyl-terminal 30 amino acids, the SH2 subdomains, and the carboxyl-terminal 68 amino acids including the SH2 subdomains deleted. The reconstitution studies of IL-2 receptors with these mutants and IL-2Rβ demonstrated that the region containing the SH2 subdomains is essential for induction of cell growth and expression of three protooncogenes, *c-myc*, *c-fos*, and *c-jun*, mediated by IL-2 (35, 90). While the region containing the carboxyl-terminal 30 amino acids participates in induction of c-fos and c-jun, it does not for c-myc and cell growth (35, 90). These observations suggest the possible existence of at least two distinct signal transducing pathways from IL-2 receptor, one for induction of c-myc, which correlates with cell growth, and the other for induction of c-fos/c-jun. The region containing the SH2 subdomains is associated with Jak3 tyrosine kinase, the activation of which correlated with IL-2-induced cell growth (67, 90–93). In fact, transfectant clones of a mouse fibroblastoid cell line NIH3T3 with human IL-2Rβ and IL-2Rγ had little IL-2 responsiveness for cell growth, but when the clones were cotransfected with Jak3 gene, they became responsive to IL-2 for DNA synthesis, suggesting that at least Jak3 activation may contribute to signal transduction for IL-2–mediated cell growth (92).

Since the Jak3 association with IL-2Rγ is independent of the receptor complex formation with the other subunits IL-2Rα and IL-2Rβ, all the cytokines sharing IL-2Rγ are expected to induce activation of Jak3. Indeed, Jak3 is activated by stimulation with IL-4, IL-7, and IL-9 as well as IL-2 (67, 92, 93). Interestingly, not only Jak3 but also Jak1 is activated by stimulation with IL-4, IL-7, and IL-9, as well as IL-2 (67, 91, 92, 93). IL-15 is also expected to activate Jak1 and Jak3 because the IL-15 receptor complex contains both IL-2Rβ and IL-2Rγ. The association of Jak1 with IL-2Rβ was also demonstrated irrespective of their complex formation and IL-2 stimulation. Thereby, it can be predicted that Jak1 is associated with the α chains of receptors for IL-4, IL-7, and IL-9. Recently, we obtained evidence that Jak1 is directly associated with the α chains of receptors for IL-4, IL-7, and IL-9 as well as IL-2Rβ, and the Jak1 association with the receptor subunits was independent of ligand stimulation. Furthermore, using IL-2Rβ mutants in the box 1 region, which exists in the α chains of receptors for IL-4, IL-7, and IL-9 as well as other cytokine receptors, we demonstrated that the box 1 region of IL-2Rβ plays a critical role in Jak1 association. However, although transfectants with the box 1 mutant of IL-2Rβ lost the ability for Jak1 association and Jak1 activation mediated by IL-2, they

proliferated and showed activation of Jak3 in response to IL-2 (M Higuchi, H Asao, N Tanaka, M Nakamura, and K Sugamura, unpublished observations). These results indicate that Jak1 is dispensable for IL-2–mediated cell growth signaling, and activation of Jak3, which should be required for the growth signaling, is mediated independently of cross-phosphorylation with Jak1.

The Stat family proteins interacting with cytokine receptors at phosphotyrosine residues via their SH2 domains are phosphorylated by the Jak family tyrosine kinases (94). The tyrosine phosphorylated Stat proteins then form homo- or heterodimers through their SH2 domains and then migrate to the nucleus, where they act as activators for transcription of genes (95). IL-2 generally activated Stat5 in IL-2-responsive cells, and Stat3 only in PHA-activated peripheral blood cells (96–100). Stat5 and Stat3 are thought to be activated by Jak1 and/or Jak3 in IL-2-stimulated cells. In contrast with the IL-2/IL-2 receptor system, IL-4, which also activates Jak1 and Jak3, activates Stat6, which is distinct from Stat5 and Stat3 (101, 102). Such differential activation of the Stat proteins by the same Jaks may be regulated primarily by the specificity of the cytokine receptor directly interacting with the Stat proteins (95).

CAUSATIVE RELATIONSHIP BETWEEN IL-2Rγ AND HUMAN XSCID

Two-Step Diagnosis for Human XSCID

Human X-linked severe combined immunodeficiency disease (XSCID) occurs in as many as 50% of patients with primary SCID, which is characterized by severe impairment of humoral and cell-mediated immunity, which can be cured only by successful bone marrow transplantations (103, 104). Patients with XSCID have complete or profound deficiency of T cells but carry normal or slightly increased numbers of B cells. The putative gene for human XSCID is reportedly located on X chromosome q13, where the IL-2Rγ gene was mapped; furthermore, mutations of the IL-2Rγ gene derived from patients with XSCID were manifested (16). All the patients with XSCID thus far detected had mutations of the IL-2Rγ gene (10). The IL-2Rγ mutations included nonsense mutations, frameshift mutations by one and two base deletions, as well as deletions of exons, leading to truncations in the extracellular and cytoplasmic domains of IL-2Rγ. These results indicate the etiological relationship between mutations of IL-2Rγ and XSCID. Thereby, the genomic sequencing of the IL-2Rγ gene has been established as a definitive diagnostic procedure for human XSCID.

As described above, most peripheral blood cell populations of normal individuals express IL-2Rγ, indicating that expression of mutant IL-2Rγ chains

Table 1 Detection of IL-2Rγ mutations in patients with XSCID

Patient	Mutation type	Nucleotide change	Amino acid change	TUGh4 staining	Reference
1	splice/deletion	deletion of the exon 2	frameshift	–	(105)
2	deletion	7 nucleotides (284–291)	frameshift	–	(108)
3	deletion	15 nucleotides (580–594)	frameshift	–	(109)
4	deletion	GATT (830–833)	frameshift	–	our unpublished data
5	deletion	GA (971–972)	frameshift	+	(105)
6	deletion	large deletion	?	–	our unpublished data
7	nonsense	C (717) → T	Gln (235) → stop	–	(109)
8	nonsense	C (717) → T	Gln (235) → stop	–	(109)
9	nonsense	C (923) → A	Tyr (303) → stop	+	(109)
10	missense	C (481) → T	Ala (156) → Val	+	(105)
11	nonsense	C (690) → T	Arg (226) → Cys	–	(108)
12	nonsense	G (691) → A	Arg (226) → His	–	our unpublished data

can be screened with immunostaining of peripheral blood cells or EB virus-transformed B cells derived from patients with XSCID. The cells derived from 12 independent patients with XSCID were stained with anti-human IL-2Rγ mAb, TUGh4, resulting in negative staining for 9 patients (Table 1) (105–109). These results suggest that the IL-2Rγ immunostaining is useful as a simple and rapid diagnosis for most patients. Thus, we propose a two-step diagnosis for XSCID, the immunostaining with TUGh4 in the first step and the genomic sequencing of the IL-2Rγ gene.

Impairment of IL-2Rγ Function in XSCID

IL-2Rγ cDNA clones derived from three patients with XSCID were isolated, and the IL-2Rγ chains encoded by the mutant genes were examined for their ability to form the functional IL-2 receptor (105). The first patient lacked the second exon in IL-2Rγ mRNA, the second (AV mutant) showed Ala^{156} substitution to Val in the extracellular domain, and the third (tSH mutant) had a two-base deletion causing a frameshift of the coding region in the SH2 subdomains in the cytoplasmic domain. It can be easily anticipated that the first mutant will have no ability to form the functional receptor complex. Analysis was then carried out with the other two mutants, which were stably introduced into a unique human T cell line, $ED40515^{-}$ (105). This cell line is useful for analysis of the IL-2Rγ function because it expresses both IL-2Rα and IL-2Rβ, but little or no IL-2Rγ (110). The transfectants with either AV or tSH mutant showed no response to IL-2 in terms of cell proliferation and induction of

protooncogenes, unlike the transfectants with the wild IL-2Rγ gene. The AV mutant was further found incapable of forming the receptor complexes for IL-4 and IL-7 or for IL-2 (N Ishii, T Takeshita, M Higuchi, and K sugamura, unpublished data). The Ala^{156} of IL-2Rγ is thought to be located in the hinge region of two fibronectin type III-like domains, the N and C domains, as expected for the cytokine receptor superfamily. The transfectants with tSH mutant showed significant binding of IL-2, which is consistent with the previous study that the cytoplasmic domain of IL-2Rγ does not affect the IL-2 binding affinity of the receptor (105). Since the tSH mutant lacks a part of the SH2 subdomains, which is essentially involved in the intracellular signal transduction, it should lose the signal transducing function. Other patients with XSCID carrying mutations in the cytoplasmic domain of IL-2Rγ were also shown to be incapable of forming the full-fledged functional IL-2 receptor complex (67).

Normal or increased numbers of B cells and hypogammaglobulinemia are generally detected in patients with XSCID. Since the B cells in XSCID are mostly surface IgM-positive, they are supposed to be incapable of secreting immunoglobulin. Such impairment of Ig class-switching and final maturation of B cells in XSCID may also result from dysfunction of IL-2Rγ on B cells. This stems from the facts that carrier females for mutant IL-2Rγ genes were demonstrated to carry mature B cells with nonrandom inactivation of the X chromosome, and various B cell clones derived from three patients with XSCID were found to utilize the biased repertoire of the J_H segments (107, 111, 112).

The complete dysfunction of IL-2Rγ such as failure of the ligand binding or inability to perform signal transduction results in the typical phenotypes of XSCID as characterized by T and NK cell defect. However, atypical cases of XSCID have also been reported; one case showed a normal number of CD4$^+$ and CD8$^+$ T cells in the periphery, but their function was impaired, and the other case showed a reduced number of T cells and normal number of NK cells (67, 113). In the former patient, the IL-2Rγ gene was transcribed into two different mRNAs, one of which encodes the nonfunctional IL-2Rγ rather abundantly as compared with the other, which encodes the functional IL-2Rγ despite a mutation of Asp^{39} to Asn. The small amount of the functional IL-2Rγ was thought to be adequate for T cell development but not for activation. The latter patient had a mutation of Leu^{291} to Glu in the cytoplasmic domain of IL-2Rγ, although the mechanism leading to the atypical phenotypes of XSCID has not yet been clarified.

Involvement of IL-7 in Early T Cell Development

The mutations of the IL-2Rγ gene result in XSCID, of which the typical feature is a profound T cell defect in the thymus and periphery. Since IL-2Rγ is shared among receptors for at least IL-2, IL-4, IL-7, IL-9, and IL-15, these cytokines

may include a responsible cytokine(s) for early T cell development. In this consideration, the IL-2-deficient patients and the knockout mice for IL-2 and IL-4 genes provided us with important information—that neither IL-2 nor IL-4 are essential for T cell development because these patients and knockout mice have mature T cells in normal ranges in the periphery (12–14, 46–48). On the other hand, IL-7 reportedly affects growth of double negative T cells (61). We then asked, using mouse thymic organ cultures, whether IL-7 is necessary for early T cell development. When the most immature thymocytes—Pgpl$^+$/c-kit$^+$ pro-T cells with CD4$^-$8$^-$ phenotype, sorted from Day 15 fetal thymus—were cultured in lobes of fetal thymus pretreated with deoxyguanosine to eliminate preexisting lymphocytes, CD4$^+$ and CD8$^+$ T cells were detected 7–10 days later. However, simultaneous addition of blocking mAbs specific for IL-7Rα (A7R34) and IL-2Rγ (TUGm2) into the culture completely inhibited development of the double-negative Pgp1$^+$/c-kit$^+$ pro-T cells into double-positive T cells, indicating that IL-7 plays a critical role for early T cell development at least in the mouse thymic organ culture system (114). These results suggest that the IL-2Rγ mutants in XSCID accompany the dysfunction of IL-7, resulting in the defect of T cell development. As described above, the AV mutant of IL-2Rγ derived from the patient with XSCID showed no ability for binding of IL-7 as well as IL-2 and IL-4. However, there is a dissimilarity between the phenotypes of mice with IL-7 dysfunction and human XSCID. Human XSCID has a normal or increased number of B cells, while in vivo blocking of mouse IL-7 function by antibody treatment induces significant reduction of B cells in addition to T cells (114, 115). Similarly, knockout mice for IL-7 or IL-7Rα gene showed the profound defect of T and B cells (116, 117). This inconsistency has to be resolved in the future to define whether or not XSCID is solely attributable to the dysfunction of IL-7.

ANIMAL MODEL FOR HUMAN XSCID

An animal model of human XSCID is useful for investigating the occurrence of XSCID caused by mutations of the IL-2Rγ gene and development of gene therapy for human XSCID. Knockout mice for the IL-2Rγ gene were recently developed, and they showed significant reduction of T, B, and NK cells (118, 119). Their phenotypes, which are not exactly identical to those of human XSCID with regard to B cell development, are quite similar to those of IL-7-dysfunctional mice. They are completely null for expression of IL-2Rγ on cell surface. However, since a couple of cases of human XSCID lack the cytoplasmic region of IL-2Rγ, they are expressed on cell surface, and they work for ligand binding but not signal transduction. Therefore, we developed, by means of gene targeting, mice expressing IL-2Rγ but lacking its cytoplasmic

domain. Our mutant mice also showed profound loss of T and B cells, and no NK cells, but in contrast to the null mutant mice, the number of monocytes was increased in our mutant mice (120). Interestingly, they had $CD34^+$/c-kit^+/$Sca1^+$ hematopoietic stem cells in spleen more than ten times as often as the control, and they carried lymphoadenopathy of celiac lymphnodes. Such increase of the stem cells was seen in our mutant mice but not in the null mutant mice, suggesting that traps of the cytokines sharing IL-2Rγ might be involved in this phenomenon (121). In humans, patients with the primary and secondary immunodeficiencies have a high risk of developing lymphomas; therefore, the lymphoadenopathy observed in our mutant mice may represent a prelymphomatous state (121). Our mutant mice as well as the null mutant mice share similar phenotypes of profound loss of T, B, and NK cells, suggesting that these mutant mice are deficient for humoral, T cell–mediated, and NK cell–mediated immunities. Since the SCID strains of mice established previously have NK cell activities, the IL-2Rγ mutant mice may serve as a more desirable SCID mouse model.

FUTURE DIRECTIONS

The discovery and molecular characterization of the third component of IL-2 receptor, IL-2Rγ, have brought us a tremendous amount of knowledge that helps us to understand the structure and signal transducing functions of various cytokine receptors. These developments have made especially great contributions toward elucidation of the molecular mechanisms of human XSCID occurrence. The cytokines sharing IL-2Rγ induce their pleiotropic and redundant functions. As expected, IL-2Rγ interacts with Jak3, which can be activated by stimulation with all the cytokines sharing IL-2Rγ. Moreover, it is noteworthy that the α chains of cytokine receptors sharing IL-2Rγ, albeit different from each other, interact with the same effector molecule Jak1. Thereby, the redundancy of cytokine actions possibly resulted, in part, from sharing of receptor subunits, and in part, from sharing of the same effector molecule irrespective of receptor subunit sharing. Little is known about the signal transduction for the pleiotropy of IL-2 functions; however, the cytoplasmic domain of IL-2Rβ interacts with several other effector molecules such as Lck, Fyn, Syk, PI3 kinase, and Shc/Grb2/Sos/Ras, and Stats. Furthermore, the region containing the carboxyl-terminal 30 amino acids of the IL-2Rγ cytoplasmic domain, which is essentially involved in signal transduction for induction of c-fos and c-jun, is expected to interact with a certain effector molecule(s). Some of these effector molecules associated with the IL-2 receptor complex may contribute to the pleiotropic function of IL-2. To demonstrate the signaling pathways for the pleiotropic and redundant functions of the cytokines sharing IL-2Rγ, we

need to further investigate the downstream events of these effector molecules associated with the receptors.

The causative relationship between human XSCID and mutations of the IL-2Rγ gene has been demonstrated. Knockout mice for IL-2Rγ showed similar phenotypes to those of human XSCID, although their phenotypes were not completely the same. The difference is that B cells are significantly reduced in mouse XSCID but not in human XSCID. Similarly, the mechanism of the T cell defect in XSCID can be also explained by dysfunction of IL-7 sharing IL-2Rγ in the mouse thymic organ culture system, but dysfunction of IL-7 in mouse is known to cause the reduction of B cells. Hence, although it is strongly suggested that the IL-7 dysfunction directly leads to occurrence of XSCID in humans, it has become important to resolve the difference in usage of IL-2Rγ for B cell development between human and mouse. Apart from the precise mechanism of XSCID occurrence, the knockout mice for IL-2Rγ will provide a useful tool for the development of gene therapy for human XSCID.

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MOLECULAR MECHANISMS OF LYMPHOCYTE-MEDIATED CYTOTOXICITY AND THEIR ROLE IN IMMUNOLOGICAL PROTECTION AND PATHOGENESIS IN VIVO

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ABSTRACT

Studies with perforin-deficient mice have demonstrated that two independent mechanisms account for T cell–mediated cytotoxicity: A main pathway is mediated by the secretion of the pore-forming protein perforin by the cytotoxic T cell, whereas an alternative nonsecretory pathway relies on the interaction of the Fas ligand that is upregulated during T cell activation with the apoptosis-inducing Fas molecule on the target cell. NK cells use the former pathway exclusively. The protective role of the perforin-dependent pathway has been shown for infection with the noncytopathic lymphocytic choriomeningitis virus, for infection with *Listeria monocytogenes*, and for the elimination of tumor cells by T cells and NK cells. In contrast, perforin-dependent cytotoxicity is not involved in protection against the cytopathic vaccinia virus and vesicular stomatitis virus. LCMV-induced immunopathology and autoimmune diabetes have been found to require perforin-expression. A contribution of perforin-dependent cytotoxicity to the rejection of MHC class I–disparate heart grafts has also been observed. Its absence is efficiently compensated in rejection of fully allogeneic organ or skin

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grafts. So far, evidence for a role of Fas-dependent cytotoxicity as a T cell effector mechanism in vivo is lacking. Current data suggest that the main function of Fas may be in regulation of the immune response and apparently less at the level of an effector mechanism in host defense. Further analysis is necessary, however, to settle this point finally.

INTRODUCTION

Tumors and infections with many viruses and intracellular bacteria are serious threats to vertebrate host organisms. Because of their intracellular nature, they cannot be monitored by the immune system directly. Instead, it relies on detecting changes on the cell surface, particularly by recognizing pathogen-derived peptides presented on MHC class I molecules. Lack of direct access by soluble effector molecules and the fact that these challenges depend on the viability of the harboring cell led to the development of specialized effector cells that are able to selectively destroy infected or transformed host cells by contact-dependent cytotoxicity. Thus, the primary role of cytotoxic effector cells is to complement the function of antibodies, which are most efficient in fighting free pathogens. The importance of this effector function in protection, in immunopathology, in autoimmune diseases, and in transplant rejection has triggered an almost continous interest in the mechanisms and effector functions involved in lymphocyte-mediated cytotoxicity ever since this activity was first described (1, 2).

There are two main classes of cytolytic lymphocytes differing fundamentally in specificity: Cytotoxic T cells (CTL) recognize target cells presenting processed antigenic peptides, whereas NK cells lyse a variety of target cells without classical restriction by MHC molecules. Nevertheless, the observations that cytotoxic T cells and NK cells form conjugates with their respective target cell, possess cytolytic granules that are secreted during the interaction with the target cell (3–10), and induce the formation of lytic pores in the target cell membrane (11, 12) led to the formulation of the granule exocytosis model acounting for T cell– and NK cell–mediated cytotoxicity (13). This model was further supported by the isolation and cloning of perforin from cytolytic lymphocytes (14–19).

Perforin is a glycoprotein of 534 amino acids with sequence homology to the membrane attack complex–forming complement component C9. In analogy to C9, perforin is able to integrate in cell membranes and aggregates are forming polyperforin pores comprising 12–18 monomers of 10–20 nm internal diameter. Perforin expression is mainly confined to $CD8^+$ T cells, NK cells, and $\gamma\,\delta$ T cells (20–22), but perforin expression has been reported in a $CD4^+$ T cell clone (23)

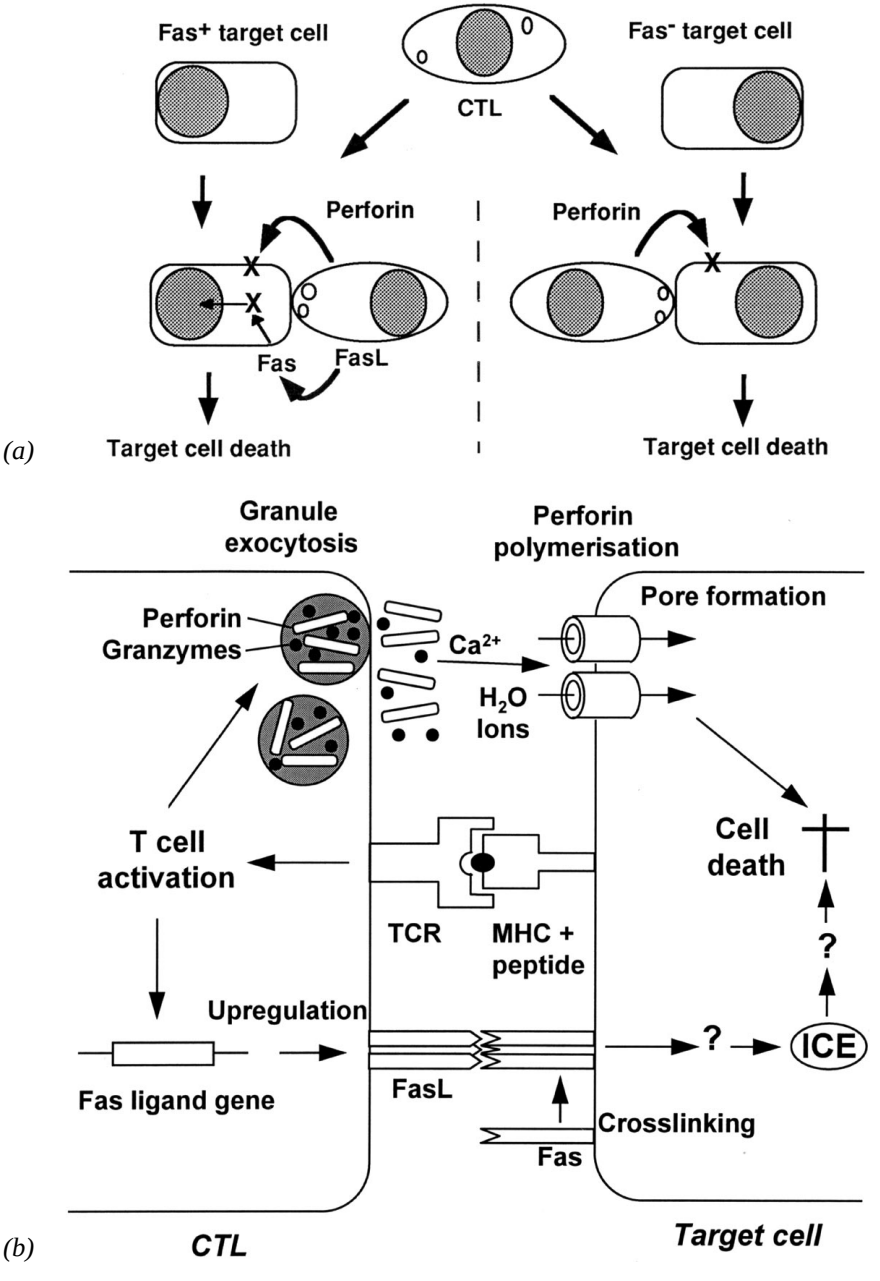
and in peripheral $CD4^+$ T cells from a patient with infectious mononucleosis (24). The expression level of perforin in vivo during a viral infection correlates with the kinetics of the cytotoxic activity (25, 26).

The granule exocytosis model proposes that, upon interaction with the target cell, the cytotoxic effector cell releases the content of its cytoplasmic granules in a directed manner into the intercellular space between lymphocyte and target cell. There, perforin undergoes a Ca^{2+}-induced conformational change, integrates into the target cell membrane, and forms pores, which are similar to the pores formed by the membrane attack complex of the complement system. The permeabilization of the target cell membrane finally leads to the death of the target cell. The effector cells recycle and are able to lyse additional target cells. Although plenty of evidence supported the granule exocytosis model, the model was not generally accepted and remained controversial (27–29). Several alternative, not necessarily mutually exclusive, effector mechanisms accounting for lymphocyte-mediated cytotoxicity were postulated, including various effector molecules such as factors homologous to TNF (30), secreted ATP (31, 32), the polyadenylate-binding protein TIA-1 (33), granule-associated serine esterases called granzymes (34–39), and the notion that cytotoxic lymphocytes induce programmed cell death in the target cell (40, 41).

Recently, the generation of perforin-deficient mice has helped to clarify much of this debate. In addition, these mice have allowed further analysis of the lytic mechanisms of CTL and NK cells and provided a tool to assess the contribution of cytotoxic versus other effector mechanisms in a number of immune functions in vivo, including antiviral and antilisterial immunity, immunopathology, graft rejection, and autoimmunity. This review summarizes some of the recent progress obtained by analyzing the phenotype of perforin-deficient mice.

MECHANISMS OF $CD8^+$ T CELL–MEDIATED CYTOTOXICITY

Evaluation of the cytotoxic activity of perforin-deficient T cells allowed us to assess the thus-far-controversial role of perforin as an effector molecule in T cell–mediated cytotoxicity. Since inactivation of perforin does not impair activation and proliferation of T cells (42), perforin-deficient mice can be used to study defects on the level of effector mechanisms without complicating indirect effects. These experiments have clearly established the perforin-dependent granule exocytosis pathway (Figure 1*b*, top) as a main cytotoxic pathway, because cytotoxic activity of $CD8^+$ T cells from perforin-deficient mice against fibroblast and certain lymphohematopoietic target cells was completely



absent (42). Most lymphohematopoietic target cells were lysed with varying but severalfold reduced efficiency by perforin-deficient T cells. This cytolytic activity was found to be mediated by the interaction of Fas ligand expressed on activated T cells with Fas on the surface of the target cell (42–46) (Figure 1*b*, below). Fas, also called Apo-1 or CD95, was originally isolated with two independent antibodies that induced apoptosis in various human cell lines (47, 48). Fas belongs to the tumor necrosis factor (TNF) receptor and nerve growth factor receptor family, and it shares with TNF receptor 1 a conserved extracellular region and a cytoplasmic domain, which is essential for the induction of apoptosis (49, 50). Fas-expressing target cells can be lysed upon interaction with Fas ligand expressed on activated T cells. The following experimental evidence supports the existence of only these two cytotoxicity pathways in $CD8^+$ T cell–mediated cytotoxicity:

(i) The relative contribution of the perforin-independent pathway of cytotoxicity, quantified by comparing the effector to target cell ratios of cytotoxicity by control and perforin-deficient effector cells in the linear range of the semilogarithmic E/T ratio vs ^{51}Cr release diagrams, correlates with the expression level of the Fas molecule on the target cell (51). Transformed and untransformed primary fibroblasts do not express Fas and are completely resistant to cytotoxicity by perforin-deficient effector cells, whereas Fas-expressing

Figure 1 Schematic representations of the two pathways in T cell–mediated cytotoxicity. (*a*) The two pathways of cytotoxicity: Fas negative target cells are lysed by the action of the perforin-dependent pathway. Fas-expressing target cells, however, are killed by the concomitant effect of the perforin- and the Fas-dependent pathway. (*b*) Steps and molecules involved in the two pathways of T cell–mediated cytotoxicity. For both pathways the engagement of the specific T cell receptor complex and T cell activation are primary events. On the one hand, the perforin-dependent granule exocytosis pathway (shown on top) is then initiated by accumulation of cytoplasmic granules containing perforin and granzymes at the interface between effector T cell and target cell, followed by directed exocytosis of these granules upon the target cell. The elevated free Ca^{2+} concentration in the extracellular space compared to the granular compartment induces a conformational change of the perforin molecules, rendering them amphipathic and able to insert into the target cell membrane. There, 10–18 perforin molecules aggregate to form polyperforin pores, which make the target cell membrane permeable to water and small ions. This permeabilization, eventually, together with effects of additional molecules such as granzymes entering through the polyperforin pore into the target cell cytoplasm, leads to death of the target cell. On the other hand, the Fas-dependent pathway (shown below) is initiated by upregulation of Fas ligand expression on the T cell. Binding and cross-linking of the Fas ligand molecule, which is most probably present on the membrane in a trimeric form, with Fas molecules on the target cell lead to the induction of apoptosis. A death-inducing cytoplasmic domain on the Fas molecule triggers an apoptosis program probably involving several interacting molecules, one of which may be interleukin-1β converting enzyme (ICE) and/or a related protease.

lymphohematopoietic target cells are lysed with varying efficiency (42). A discrepancy somewhat overstressed in a recent publication (46) reporting lysis of fibroblasts by perforin-deficient T cells may be explained by unspecific toxicity to fragile target cells at high effector-to-target cell ratios.

(ii) A Fas-specific monoclonal antibody (52) that is not cytotoxic during the 5-h incubation period of the cytotoxicity assay is able to completely block the lytic activity of perforin-deficient T cells against lymphoma target cells. Conversely, overexpression of Fas by transfection of an expression construct into a target cell line enhances the cytotoxic activity of perforin-deficient T cells (43).

(iii) Con A blast target cells from mice with the lpr mutation, which results in inactivation of the Fas molecule (53), are (in contrast to con A blast target cells from normal mice) resistant to perforin-independent cytotoxicity (43).

Thus, dependent on the target cell, $CD8^+$ T cells confer lysis by either a perforin-dependent pathway alone or by the concomitant action of the perforin-dependent and the Fas-dependent pathway (Figure 1*a*). The complete absence of cytotoxicity of perforin-deficient T cells in the presence of a blocking Fas-specific antibody or against target cells from Fas-deficient lpr mice demonstrates that these two pathways alone account for $CD8^+$ T cell–mediated cytotoxicity without any involvement of additional pathways.

Except for reduced efficiency on most target cells, killing via the Fas-dependent pathway exhibits characteristics similar to those of perforin-dependent cytotoxicity: It is blocked by anti-CD8 antibodies; it appears 6 days after LCMV infection and peaks on day 8; and it is specific for an antigenic peptide bound to MHC class I molecules (43).

One of the arguments used to challenge the role of granule exocytosis was that cytotoxicity against certain target cells was not completely abolished in the presence of EGTA, which inhibits exocytosis by binding free Ca^{2+} (29, 54). To evaluate the effect of Ca^{2+}-chelation on either the perforin- or Fas-dependent pathway separately, we evaluated the cytotoxicity either of normal CTL on Fas-negative target cells or of perforin-deficient CTL on Fas-expressing target cells, respectively. Perforin-dependent cytotoxicity was completely inhibited in the absence of free Ca^{2+}, whereas Fas-dependent cytotoxicity was only threefold reduced (D Kägi, H Hengartner, unpublished data). Thus, cytotoxicity tested in the absence of Ca^{2+} is mediated by the Fas-dependent pathway, but Fas-dependent cytotoxicity is not completely Ca^{2+}-independent, as was suggested by an early report (55).

Recently, the Fas ligand expressed on cytotoxic T cells was identified and found to be homologous to membrane TNF (56). Therefore, it is currently thought that Fas-dependent $CD8^+$ T cell–mediated cytotoxicity requires the

interaction of Fas ligand on the $CD8^+$ T cell and the Fas molecule on the target cell, and that the induced trimerization of Fas by the ligand triggers cell death by a yet unknown intracellular pathway (57) (Figure 1*b*). Of the several steps and molecules probably involved in this pathway, a step requiring the activity of interleukin-1β–converting enzyme (ICE) and/or ICE-related proteases has been recently identified (58, 59). The homology of ICE to the cell death–inducing gene *ced-3* from the nematode *Caenorhabditis elegans* suggests that Fas-induced apoptosis follows a highly conserved pathway.

Based on the observation that T cell activation induces Fas ligand expression, the specificity of Fas-dependent cytotoxicity probably results from upregulation of cell-bound Fas ligand (56) upon triggering of the T cell receptor during conjugate formation with a specific target cell. Alternatively, activated T cells may secrete soluble Fas ligand upon interaction with the target cell (60). However, the observed strict specificity of the Fas-dependent pathway, also tested in cold target inhibition assays, would require an extremely rapid inactivation of secreted Fas-ligand.

So far, the concept involving two independent pathways in CTL-mediated cytotoxicity has been confirmed for a number of different cytotoxic $CD8^+$ T cell populations, including H-2 alloantigen–specific CTL generated in mixed lymphocyte cultures in vitro, in vivo–activated antiviral and H-2 alloantigen–specific spleen cells, and H-2 alloantigen–specific peritoneal exudate lymphocytes (42). The finding that for all of the tested effector cells the relative contribution of the two cytotoxic pathways depends similarly on the expression of Fas by the target cell, and not on the type of the effector cell, resolves the long-standing debate (27) about whether peritoneal exudate lymphocytes possess cytotoxic mechanisms differing from the mechanisms of cloned or splenic T cells. Although peritoneal exudate lymphocytes may express lower amounts of perforin and display less prominent cytoplasmic granules than do cytotoxic T cell clones, perforin-dependent cytotoxicity is apparently fully functional.

Besides lysis of the plasma membrane as detected by release of ^{51}Cr, CTL also induce fragmentation of target cell DNA into multiples of nucleosomal 200 bp fragments. Whereas ^{51}Cr-release is only detected 1 h after mixing effector and target cells, DNA fragmentation is detected more rapidly (40, 41). It was difficult to bring this observation into agreement with the concept that membrane disruption by perforin pores directly induces cell death, because disruption of the membrane by complement lysis or chemical compounds does not induce DNA fragmentation. Recent experimental evidence suggests that granzymes may be co-secreted with perforin and enter the target cell through the polyperforin pore to trigger a target cell internal pathway that results in

DNA fragmentation (36, 61). The following lines of evidence support this notion: (i) A noncytotoxic rat mast cell tumor line is rendered cytotoxic by transfection with an expression construct for perforin. This cytotoxicity as well as the capacity to induce DNA fragmentation is enhanced by the additional expression of granzyme A (62, 63). (ii) Cytotoxic T cells from granzyme B–deficient mice exhibit a reduced capacity to induce DNA fragmentation in tumor target cell lines (64).

Further investigations are necessary to clarify which granzymes or other granular proteins are involved in cytotoxicity and whether they are required for perforin-dependent effector functions in vivo such as e.g. clearance of LCMV infection.

MECHANISMS OF CYTOTOXICITY BY CD4$^+$ T CELLS

Cytolytic CD4$^+$ T cells can be generated by a number of protocols, often involving depletion of CD8$^+$ T cells (65–69). Their MHC class II–restricted cytotoxic activity is readily measured in vitro. Whether they mediate a cytolytic effector or an immunoregulatory function in vivo, however, is unclear. Different target cells vary in their susceptibility to CD4$^+$ T cell–mediated cytotoxicity; the B cell lymphoma A20 and LPS-activated B cell blasts are preferentially used for testing the cytolytic activity of CD4$^+$ T cells due to their sensitivity, whereas fibroblast target cells transfected with MHC class II molecules are relatively resistant (70). Recent experiments have explained this observation by showing that unspecifically activated or redirected splenic CD4$^+$ T cells and CD4$^+$ T cell clones require the expression of Fas by the target cell for lytic activity (71–73). Stalder et al (72) interpreted the reduction of CD4$^+$ T cell–mediated cytotoxicity observed upon addition of EGTA as an indication of involvement of the perforin-dependent pathway. However, since it is established now that Fas-dependent cytotoxicity is not completely independent of free Ca^{2+}, this observation would also accord with an exclusive role for the Fas-dependent pathway.

Recently, it was found that anergic autoreactive B cells can be eliminated by CD4$^+$ T cells of the same specificity in a Fas-dependent manner (74). This suggests an immunoregulatory function of CD4$^+$ T cell–mediated Fas-dependent cytotoxicity.

Thus, current evidence indicates that the Fas-dependent pathway plays a more prominent role in CD4$^+$ than in CD8$^+$ T cell–mediated cytotoxicity; CD4$^+$ T cells may even lyse exclusively via the Fas-dependent pathway. This latter point should be clarified readily by testing the cytotoxic activity of nominal antigen- or MHC class II alloantigen–specific polyclonal CD4$^+$ T cells from normal control, perforin-deficient, and Fas ligand–deficient gld mice.

CYTOTOXICITY BY NATURAL KILLER CELLS

The notion that NK cells use the same effector mechanisms as $CD8^+$ T cells was supported by the detection of similar cytoplasmic granules containing perforin in NK cells and $CD8^+$ T cells, the acquisition of NK-like specificity of cloned $CD8^+$ T cells, and the ability of CTL and NK cells to secrete granule contents upon conjugate formation. Recently, NK cell receptor molecules have been characterized that confer some specificity to the activity of monoclonal NK cells by transmitting an inhibitory signal upon recognition of MHC class I (75–80). The results indicate that NK cells recognize either the absence or major changes of MHC class I antigens as they might be present in tumor cells or in cells infected by viruses or bacteria.

In normal control mice NK activity is readily detected after injection of poly-IC or early after LCMV infection, before measurable T cell–mediated cytotoxicity has developed. Use of these protocols that seem to activate NK cells by stimulating interferon secretion reveals that NK cells from perforin-deficient mice lack cytotoxic activity on the NK cell–sensitive target cell line YAC-1 (42) and on the MHC class I–negative lymphoma RMA-S (81). Since YAC-1 target cells express high levels of Fas and are very sensitive to Fas-dependent cytotoxicity by alloreactive $CD8^+$ T cells (51), the results indicate that NK cells utilize only the perforin-dependent pathway of cytotoxicity. The failure of NK cells to express Fas-dependent cytotoxicity requires further analysis but may be due to a lack of functional Fas ligand expression.

THE ROLE OF LYMPHOCYTE-MEDIATED CYTOTOXICITY AS AN EFFECTOR FUNCTION IN VIVO

Perforin-deficient mice allow us to assess directly the role of lymphocyte-mediated cytotoxicity in vivo, which is thought to be a crucial effector mechanism in immune protection against viruses, intracellular bacteria, and tumor cells. On the other hand, cytotoxicity may cause pathology in rejection of foreign organ transplants, autoimmune diseases, and immunopathology. So far, evidence that indeed cytotoxicity and not other effector mechanisms is responsible for these phenomena has been mostly circumstantial. Cytotoxicity has usually been claimed to account for an immunological phenomenon when cytotoxic activity was detectable, sometimes only after one or several restimulations in vitro, or when depletion of $CD8^+$ or NK marker positive cells resulted in reduction of protection or pathology. However, detection of cytotoxic activity does not necessarily mean that it is functionally effective and responsible for an observed effect. Furthermore, a requirement for $CD8^+$ or NK cells does not prove that these cells exert their function by cytotoxicity and not via secreted cytokines.

Perforin-deficient mice possess normal numbers of CD8 and NK marker positive cells, and $CD8^+$ T cells are activated and proliferate normally in response to LCMV infection (42). This shows that lymphocyte development was not affected by the lack of perforin and that indirect effects of the perforin gene disruption are absent. Thus, perforin-deficient mice are a useful tool to study the role of contact-dependent cytotoxicity vs. soluble factors in a wide range of different physiological situations in vivo.

Infection with Cytopathic and Noncytopathic Viruses

$CD8^+$ T cells recognize peptides derived from intracellularly synthesized antigens, which are presented on the almost ubiquitously expressed MHC class I molecules and are therefore thought to play a key role in defense against intracellular pathogens such as viruses. As virus-specific cytotoxic T cells are induced upon infection with many different viruses, it has been assumed that T cells prevent virus proliferation by lysing infected cells. Theoretically, lysis of an infected cell by cytotoxic T cells could occur in the eclipse phase before any infectious virus has been produced and therefore could inhibit virus replication completely (Figure 2). Alternatively, cytotoxic T cells may just reduce the production of infectious virus from an infected cell by lysing it at a certain time point after assembly and release of infectious virions has already started. If recognition by cytotoxic T cells and lysis is relatively slow compared to the cytopathic effect of a virus, then cytotoxic activity is effective only against noncytopathic viruses, and the primary role of CTL is to eliminate otherwise persisting virus-releasing cells from the organism. If T cell–mediated cytotoxicity, on the other hand, is quick relative to the cytopathic effect, then it may prevent the generation of infectious viruses completely by lysing the infected cell in the eclipse phase, or at least reduce the infectious virus yield produced by an infected cell (82). Thus, the relative kinetics of cytotoxic activity in vivo and the cytopathic effect of a virus determine whether cytotoxicity is effective only against noncytopathic or equally against cytopathic and noncytopathic viruses.

The notion that $CD8^+$ T cells control virus infections by cytotoxicity has been questioned repeatedly (41, 83–85). Instead, it was suggested that T cells control virus infections by the secretion of antiviral lymphokines (85) or by inducing fragmentation of viral DNA without necessarily killing the target cell (prelytic halt of viral replication) (41). These questions can now be addressed more directly by testing perforin-deficient mice in various viral systems.

So far, the analysis of infection with noncytopathic LCMV or cytopathic vaccinia virus has shown that perforin-dependent cytotoxicity is crucially involved in control of acute LCMV (42) but not of vaccinia virus infection (86), although both viruses induce very strong primary cytotoxic T cell responses. Whereas in normal control mice, LCMV virus reaches maximal titers on day

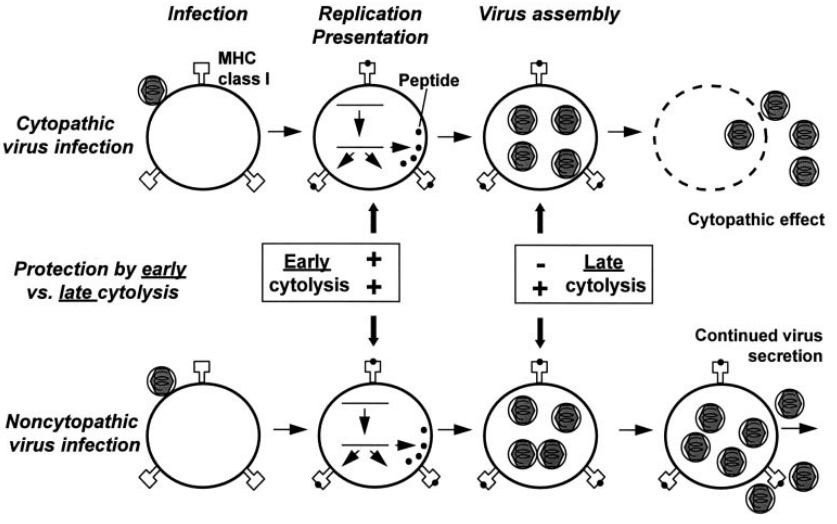


Figure 2 Control of cytopathic and noncytopathic virus infection by cytotoxic T cells. Lysis of an infected cell may occur in the eclipse phase, when the cell has been infected by the virus but infectious virions have not been formed yet, or in mid-cycle, when infectious virions have already been formed but the full virus yield has not been produced yet. Either case theoretically should lead to protection in infections with cytopathic and noncytopathic viruses. In contrast, lysis relatively late in the infectious cycle protects only in the case of noncytopathic viruses; there, an infected cell may persist and continuously release infectious virions over longer time periods if elimination by cytotoxic T cells fails. Late lysis in the case of infection with a cytopathic virus, however, does not reduce viral spreading, because a maximal yield of infectious virus may have already been assembled and will be released upon cytolysis. It has been observed that noncytopathic LCMV infection is controlled by perforin-dependent cytotoxicity, whereas in infection with cytopathic vaccinia, vesicular stomatitis or Semliki Forest virus, perforin is not measurably involved in protection. This indicates that recognition and lysis of infected host cells by CTL occur too late in the infectious cycle to be effective against cytopathic viruses.

four to six and is eliminated by $CD8^+$ T cells by day eight, in perforin-deficient mice, LCMV is not cleared and high titers persist beyond day eight (42). Because LCMV is not cytopathic in mice, it is expected that failure to control LCMV would result in persistent infection without obvious disadvantage to the mouse, similar to the virus carrier state in neonatally LCMV-infected mice (87, 88). However, perforin-deficient mice progressively lose body weight and eventually die between day 12 and day 20. Because persistent infection with high viral titers in all organs causes no obvious symptoms in normal mice, this LCMV-induced wasting disease is probably of an immunopathological

Table 1 Role of cytotoxicity in infection with various pathogens

Pathogen	Property	Protecting cell types	Role of cytotoxicity in protection: Perforin pathway	Fas pathway
LCMV	Noncytopathic	$CD8^+$ T cells	Crucial	Not involved
Vaccinia virus	Cytopathic	$CD4^+$ and $CD8^+$ T cells	Not involved	Not involved
VSV	Cytopathic	$CD4^+$ and B cells (IgG)	Not involved	Not involved
Listeria	Intracelluar bacteria	Macrophages, $CD4^+$ and $CD8^+$ T cells, granulocytes, NK cells, $\gamma\delta$ T cells	Mediates protection by $CD8^+$ T cells in secondary and late in primary infection	Not tested

nature. Up to now, the cause of death has been unclear as well as whether immunopathology is caused by Fas-dependent cytotoxicity against virus infected cells or by toxic doses of cytokines secreted by T cells that are constantly stimulated by confrontation with virus infected cells.

LCMV infection induces not only perforin-dependent but also Fas-dependent LCMV-specific cytotoxicity (43). Obviously, perforin-dependent cytotoxicity is necessary to resolve LCMV infection, and Fas-dependent cytotoxicity alone is not sufficient. Since LCMV infects Fas-positive cells such as macrophages and hepatocytes but also Fas-negative cells such as epithelial cells, a potential antiviral effect of Fas-dependent cytotoxicity is confined to virus-infected, Fas-positive cells. For complete viral clearance, however, virus has to be eliminated from Fas-positive as well as from Fas-negative infected cells. Alternatively, as a result of selection, LCMV may have developed mechanisms causing resistance to Fas-mediated lysis by the expression of apoptosis-inhibiting factors in the infected cells. Fas-mediated apoptosis is an active suicide process of the target cell and may offer more possibilities of interference than does perforin-dependent lysis. Recently, the product of the *crmÀ* gene from cowpox virus has been shown to inhibit Fas-mediated apoptosis (58, 59, 89–91).

Apparently, Fas-dependent cytotoxicity is not sufficient to clear LCMV infection. Although Fas-deficient lpr (53) and Fas ligand–deficient gld mice (92, 93), both lacking Fas-dependent cytotoxicity, clear LCMV normally (86), a certain contribution of Fas-dependent cytotoxicity to viral resistance is theoretically conceivable. Such an antiviral effector function would be most readily detected in perforin-deficient mice, where the absence of the main effector mechanism would facilitate the detection of secondary mechanisms. Therefore, we reasoned that an increase of the LCMV-specific precursor T cell frequency in perforin-deficient mice would result in enhanced LCMV-specific,

Fas-dependent cytotoxicity. This was achieved by two protocols leading to protection in normal mice, namely, by expression of a TCR transgene from a LCMV-specific $CD8^+$ T cell clone (94, 95) or by immunization with a recombinant vaccinia virus expressing LCMV glycoprotein (96, 97). If Fas-dependent cytotoxicity had a protective effect against LCMV, the increased precursor frequency would result in lower viral titers in perforin-deficient animals. However, TCR transgenic or recombinant vaccinia virus–primed perforin-deficient mice had titers that were not significantly different from titers in nontransgenic unprimed perforin-deficient mice (86); this indicates that Fas-dependent cytotoxicity is not measurably involved in specific T cell–mediated protection against LCMV infection.

Previous studies established that $CD8^+$ T cells are not necessary to clear vaccinia virus infection and that secretion of IFN-γ and TNF-α by $CD4^+$ and $CD8^+$ T cells is involved in immunity against pox viruses (98–103). Because perforin-deficient, Fas-deficient lpr, Fas ligand-deficient gld, and normal control mice eliminate intravenous and intradermal infection with vaccinia virus with comparable efficiency, perforin-dependent as well as Fas-dependent cytotoxicity are not measurably involved in clearance of vaccinia virus infection (86).

To investigate infection with other cytopathogenic viruses, we tested the survival of normal control, Fas-, Fas ligand-, and perforin-deficient mice after infection with a high dose of vesicular stomatitis virus (VSV), or Semliki Forest virus (SFV), which are both lethal to young and IFN-α/β receptor–deficient mice (104). All four groups of mice survived VSV or SFV infection without any apparent symptoms, indicating that neither perforin- nor Fas-dependent cytotoxicity is crucially involved in control of these two virus-infections (86). Infection with VSV leads to an extensively studied prompt virus-neutralizing antibody response (105–107). Together with the restriction of viral replication by the interferon system very early in infection (104, 108), neutralizing antibodies are predominantly responsible for the control of VSV infection.

The results with LCMV, vaccinia virus, VSV, and SFV infection may indicate a fundamental difference in how T cells are controlling cytopathic versus noncytopathic virus infections. Whereas in infections with noncytopathic viruses, destruction of the infected cell by perforin-dependent cytotoxicity is of prime importance, cytopathic viruses such as vaccinia virus or VSV are apparently more efficiently controlled by reducing the infection of neighboring cells by soluble cytokines or neutralizing antibodies. The failure to detect a role of both cytolytic pathways in protection against vaccinia virus infection is compatible with the notion that recognition and lysis of infected cells by CTL is too slow to achieve lysis in eclipse or mid-phase, which would be necessary

to reduce the number of virus progeny produced by a cell infected with a cytopathic virus.

Infection with Intracellular Bacteria

Immunity to intracellular bacteria is conferred by various cell types including activated macrophages (109), NK-cells (110), TCR γ/δ T cells (111), granulocytes (112), and $CD4^+$ and $CD8^+$ T cells (113–116). Interferon-γ (117–119) and tumor necrosis factor (120, 121) play essential roles in this defense. It is widely held that the main function of T cells in infection with intracellular bacteria is to activate bactericidal macrophages by the secretion of IFN-γ (109).

T cells with the capacity to confer specific cytotoxicity after restimulation in vitro have been isolated from mice after infection with various intracellular bacteria. Such $CD8^+$ cytolytic T cell lines were used successfully to protect against bacterial infection by adoptive transfer (122). Injection of IFN-γ neutralizing antibodies failed to inhibit the protective effect of $CD8^+$ T cell clones, suggesting that $CD8^+$ T cells may be involved in antibacterial immunity not only by secretion of IFN-γ but also by direct contact-dependent cytotoxicity (123).

To assess the role of T cell–mediated cytotoxicity in a well-established murine model system for immunity against intracellular bacteria, we evaluated resistance of perforin-deficient mice against *Listeria monocytogenes*. In protection from secondary infection as well as in adoptive transfer experiments, perforin-dependent cytotoxicity accounted for most of the protective effect of $CD8^+$ T cells (124). $CD8^+$ T cells from perforin-deficient mice were about 100-fold less protective than $CD8^+$ T cells from normal control mice. In primary infection, however, perforin-deficient mice were able to clear Listeria, indicating that the impaired anti-Listeria effector function of $CD8^+$ T cells is quite efficiently compensated by other cell types and effector mechanisms. A significant delay of Listeria clearance from the spleen but not from the liver of perforin-deficient mice indicated that compensation was not complete. Similar to nude and scid mice, activated macrophages may also persist in perforin-deficient mice and cause reduced Listeria titers very early after infection, due to natural innate resistance. Whether other cell types also contribute to this compensatory phenomenon is not clear.

Thus, perforin-dependent cytotoxicity is a main effector mechanism exerted by $CD8^+$ T cells in immunity to secondary Listeria infection and in the later phase of the primary immune response. This conclusion is also supported by the study of β2-microglobulin-deficient mice, in which the lack of $CD8^+$ T cells results in delayed clearance of Listeria as in perforin-deficient mice (116). The protective effect of perforin-dependent cytotoxicity seems to be more significant in the spleen than in the liver. Whether this reflects restricted accessibility by $CD8^+$ T cells to the liver remains to be established.

Tumor Surveillance

MHC class I–positive, specific tumor antigen–expressing tumor cells are controlled by T cell–mediated adaptive immunity (125, 126). Tumor cells may, however, escape this immune surveillance by downregulating the expression of MHC molecules. It has recently been shown that NK cells complement T cells in immune surveillance in that growth of MHC class I–negative tumor cells is controlled by NK cell–mediated natural immunity (77, 127–129). So far it has not been defined for either effector cell population whether tumoricidal activity is exerted by contact-dependent cytotoxicity or by secreted lymphokines such as tumor necrosis factors.

To address this question, we evaluated the elimination of injected tumor cells in perforin-deficient and normal control mice. T cell–mediated tumor rejection was tested with syngeneic methylcholanthrene-induced MC57G fibrosarcoma cells (130). Perforin-deficient mice are significantly more susceptible to these fibrosarcoma cells such that, in perforin-deficient mice, doses of MC57G cells that are ten to a hundred times lower than in normal mice lead to growth of fibrosarcomas, indicating an important role of perforin-dependent cytotoxicity in tumor surveillance by T cells (42). Resistance of perforin-deficient mice to low doses of fibrosarcoma cells is probably not mediated by the Fas-dependent pathway, because the MC57G cells do not express Fas in vitro and are resistant to cytotoxicity by perforin-deficient effector cells. Whether other Fas-expressing tumor cells are controlled by T cell–mediated Fas-dependent cytotoxicity in vivo remains to be established.

To investigate the role of perforin in controlling the growth of MHC class I–negative tumors by NK cell–mediated natural immunity, we took advantage of a model system established by Kärre et al (128), using MHC class I–expressing and –nonexpressing variants (RMA-S) of the T cell lymphoma RBL-5 induced by Rauscher murine leukemia virus. This group demonstrated that growth of injected MHC class I–negative RMA-S is restricted by natural immunity (128). We evaluated resistance against RMA-S cells and found that perforin-deficient mice were sensitive to an at least 10-fold lower dose of RMA-S than were C57BL/6 control mice (81). Thus, perforin-dependent cytotoxicity is crucially involved in control of tumor cell growth mediated by T cells as well as by NK cells.

Immunopathology

Upon infection with intracellular pathogens, an infected host generates in most cases a strong immune response leading to quick clearance of the pathogen. Pathogens such as hepatitis B virus, LCMV, and *mycobacterium leprae* rarely succeed in establishing a carrier state, which is characterized by the absence

of an effective immune response and a relatively low toxicity of the pathogen itself. Between these two extremes, however, when a noncytopathic pathogen persists, and the immune response is not completely inhibited, the continued confrontation of T cells and virus-infected cells can result in immunopathology due to damage of host tissue. A better understanding of the immunological effector mechanisms leading to tissue damage may help to improve the therapy of such conditions.

A classical model for virus-induced immunopathology is the lethal lymphocytic choriomeningitis that develops in normal mice but not in mice lacking T cells upon i.c. infection with LCMV (131–133). The disease is characterized by infection of ependymal, meningeal, and choroid plexus cells and by a marked cerebral infiltration of $CD4^+$ and $CD8^+$ T cells. It has been widely debated whether lethal pathology is due to cytotoxic T cells lysing infected leptomeningeal or neuronal cells, due to cytokines interfering with synaptic functions, or due to the increase of intracranial pressure from edema. The observation that perforin-deficient mice fail to develop the characteristic symptoms of lymphocytic choriomeningitis (42) but nevertheless display marked infiltration of $CD8^+$ and $CD4^+$ T cells into the brain (D Kägi et al unpublished observation) indicates that immunopathology during LCMV-induced choriomeningitis is due to lysis of infected leptomeningeal or neuronal cells by perforin-dependent cytotoxicity, which is exerted by infiltrating $CD8^+$ T cells.

Viscerotropic isolates of LCM virus induce immunopathological hepatitis when injected at high doses intravenously. The destruction of infected liver cells by $CD8^+$ T cells is conveniently monitored by measuring the serum level of liver-specific transaminases or glutamic acid dehydrogenase. LCMV-specific $CD8^+$ T cells cause the release of these enzymes from infected liver cells (134), and the infiltrating mononuclear cells express perforin (25).

Although $CD8^+$ T cells infiltrate the liver of perforin-deficient mice to an extent similar to that in normal mice, liver enzymes do not increase (42). Thus, hepatocyte destruction occurring upon intravenous injection of high doses of LCMV is caused by $CD8^+$ T cell–mediated, perforin-dependent cytotoxicity. This model also constitutes an experimental system to test cytotoxicity of virus-specific T cells in vivo. Instead of a test tube to incubate effector cells and target cells as in the ^{51}Cr release assay in vitro, effector and target cells interact in the physiological environment of the liver, and, instead of ^{51}Cr release, release of liver-specific enzymes into the serum is measured. Taken together, this in vivo correlate of the in vitro ^{51}Cr release test not only shows the involvement of perforin-dependent cytotoxicity in immunopathological damage to host tissue but also confirms the crucial role of perforin for $CD8^+$ T cell–mediated cytotoxicity in vivo.

Autoimmune Diabetes

Animal models of diabetes have provided convincing evidence that development of diabetes is caused by a breakdown of T cell tolerance and depends on T cell function (135–139). The relative contribution of $CD4^+$ and $CD8^+$ T cells to initiation and progression of the disease is controversial (140–148). Similarly, the immunological mechanisms leading to the destruction of insulin-producing β-cells in the pancreas are unclear; β-cell damage may either result from direct cytotoxic activity of autoreactive $CD8^+$ T cells or be the consequence of the high sensitivity of β-cells to cytokines and/or reactive oxygen products, which are released during a local inflammatory process induced by β-cell–specific $CD4^+$ T cells infiltrating into the islets (149).

To test the former hypothesis, we used transgenic mice expressing the glycoprotein from lymphocytic choriomeningitis virus (LCMV-GP) exclusively in the β-cells of the pancreas (150). In this well-characterized model system, LCMV-GP-specific T cells are not tolerized but are normally reactive. Therefore, the activation of LCMV-GP transgene-specific T cells by LCMV infection induces islet destruction and diabetes within 10 to 12 days. In the islet infiltrates, $CD8^+$ T cells dominate and are observed throughout the islets. $CD4^+$ T cells, infiltrating in lower numbers than $CD8^+$ T cells, are mainly found peri-insularly. To test the role of perforin-dependent cytotoxicity in this model system, we generated perforin-deficient LCMV-GP transgenic mice. In such mice, LCMV infection does not induce diabetes, despite the infiltration of $CD8^+$ T cells into the pancreatic islets (D Kägi, PS Ohashi, R Zinkernagel, H Hengartner et al, manuscript in preparation). This indicates that perforin-dependent cytotoxicity is responsible for the rapid and efficient destruction of the majority of insulin producing β-cells, necessary to generate hyperglycemia. However, the findings do not exclude an involvement of perforin-independent effector mechanisms causing a limited degree of β-cell damage, which may be functionally compensated by the remaining β-cells.

Graft Rejection

Rejection of allogeneic tissue grafts in general is mediated by T cells, except in rejection of xenografts and in some situations where preformed anti-bloodgroup or anti-MHC specific antibodies are present in the recipient. During rejection, grafts are normally infiltrated by NK cells, macrophages, and $CD4^+$ and $CD8^+$ T cells. Although the role of $CD4^+$ and $CD8^+$ T cells in graft rejection is still the subject of controversy, experiments in mice suggest that $CD4^+$ T cells recognize MHC class II differences between graft and host whereas $CD8^+$ T cells recognize MHC class I (151). This has been concluded from studies showing that rejection of grafts with only an MHC class I or class II difference is

controlled exclusively either by $CD8^+$ or by $CD4^+$ T cells (152, 153). Due to the remarkable specificity of rejection, which results in survival of only syngeneic, but not allogeneic, cells in transplantations of skin from tetraparental mice (154–156), and because T cells with potent alloreactive cytotoxic activity are readily generated in vitro and during rejection, it has been assumed that $CD8^+$ T cells mediate graft rejection by contact-dependent cytotoxicity. Transplantation experiments with perforin-deficient mice confirmed this hypothesis only partially: Heart grafts differing in MHC class I antigen only (H-2 K^{bm1} grafts into H-2 K^b recipients) are rejected significantly more slowly by perforin-deficient than by normal mice (157). Fully allogeneic ectopic heart implants, however, are rejected with the same kinetics as by perforin-deficient and normal control mice. Skin grafts with a full allogeneic difference as well as skin grafts differing only in MHC class I alloantigens are rejected comparably by perforin-deficient and normal control animals (D Kägi, H-R Ramseier, H Hengartner, R Zinkernagel et al, unpublished observation). Thus, perforin-dependent cytotoxicity is involved in rejection by MHC class I–reactive $CD8^+$ T cells, but in the case of skin grafts and in rejection of fully allogeneic heart grafts, lack of perforin-dependent cytotoxicity is apparently compensated by other, yet undefined mechanisms. Since allogeneic grafts from lpr mice carrying an inactivated Fas gene are rejected by perforin-deficient recipients without measurable delay compared to allogenic grafts with an intact *Fas* gene, Fas-dependent cytotoxicity is not crucially involved in rejection by perforin-deficient recipients either (157). Thus, rejection by secreted cytokines and other cytotoxic factors, probably including TNF and IFN-γ, or by other inflammatory mechanisms such as e.g. delayed-type hypersensitivity response, may play a more significant role than has been assumed so far. The complexities of graft rejection, which also include revascularisation, are apparently also reflected at the level of effector mechanisms and may be due to the extraordinary strength of the alloresponse and the recognition of yet-poorly-understood minor transplantation antigens.

CONCLUSIONS

Experiments with perforin-deficient mice have brought considerable progress in our understanding of lymphocyte-mediated cytotoxicity: It is clear now that only two independent pathways, one perforin-, the other Fas-dependent, are responsible for T cell–mediated cytotoxicity. NK cells use the former pathway only. Perforin-deficient mice are also a valuable approach to characterize the role of cytotoxicity vs. other effector functions such as lymphokine secretion directly in vivo. This type of analysis has already shown the role of perforin-dependent cytotoxicity in antiviral immunity, resistance to intracellular bacteria, tumor surveillance, immunopathology, transplant rejection, and autoimmune

diabetes. Evidence for an involvement of Fas-dependent cytotoxicity as an effector mechanism in these processes, on the other hand, is still lacking. Thus, current data suggest that the main function of Fas may be in regulation of the immune response and less at the level of host defense.

In the future, perforin-deficient mice may provide a basis to further characterize the role of the two pathways of cytotoxicity, particularly in the immune response against viruses and other microbial pathogens. The evaluation of infection with LCMV, vaccinia virus, VSV and SFV suggests that T cell–mediated cytotoxicity is protective only against noncytopathic and not against cytopathic viruses. The outcome of the analysis of infections with further viruses will show whether this hypothesis applies in general. For both of the cytolytic mechanisms, additional molecules (e.g. granzymes, TIA-1, kinases) required in the respective pathway and their function remain to be characterized. Since a number of in vivo effects that are dependent on cytotoxicity are now identified, the relevance of such molecules can be tested not only in vitro but also directly in vivo.

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CD28/B7 SYSTEM OF T CELL COSTIMULATION

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ABSTRACT

T cells play a central role in the initiation and regulation of the immune response to antigen. Both the engagement of the TCR with MHC/Ag and a second signal are needed for the complete activation of the T cell. The CD28/B7 receptor/ligand system is one of the dominant costimulatory pathways. Interruption of this signaling pathway with CD28 antagonists not only results in the suppression of the immune response, but in some cases induces antigen-specific tolerance. However, the CD28/B7 system is increasingly complex due to the identification of multiple receptors and ligands with positive and negative signaling activities. This review summarizes the state of CD28/B7 immunobiology both in vitro and in vivo; summarizes the many experiments that have led to our current understanding of the participants in this complex receptor/ligand system; and illustrates the current models for CD28/B7-mediated T cell and B cell regulation. It is our hope and expectation that this review will provoke additional research that will unravel this important, yet complex, signaling pathway.

INTRODUCTION

One of the fundamental elements of the immune system has been the evolving strategies for exquisite self/nonself discrimination. The T cell receptors (TCR) and B cell receptor (BCR) play a major role in defining the fine specificity of an immune response. However, other mechanisms have been adapted to protect the

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body from untoward autoimmune reactions and to focus the immune response on the infected target tissue. These mechanisms include a unique architectural structure of the lymphoid tissue, the evolution of professional antigen presenting cells (APCs), localized antigen deposition, and homing receptors that bring the relevant immune cells to the inflammatory sites. Another mechanism that has developed as both a backup and amplification system has been the requirement for two distinct signals for T cell and B cell activation (1, 2). One signal originates from the ligation of the T cell receptor (TCR) complex and its co-receptors (e.g. CD4 and CD8). The second signal is dependent on either soluble factors such as IL-2 or the ligation of cell surface molecules that provide essential costimulatory signals complementary to the TCR engagement (3, 4). The costimulatory interactions are necessary for effective lymphocyte activation and also serve to enhance the immune response.

It is now accepted that a major T cell costimulatory pathway involves the CD28 molecule. CD28 interactions with the B7 family of costimulatory ligands are essential for initiating antigen-specific T cell responses, upregulating cytokine expression and promoting T cell expansion and differentiation. However, as is evident in this review, we have just begun to understand the complexity of the CD28/B7 costimulatory pathway.

THE CD28/CTLA-4 GLYCOPROTEINS

Expression of CD28 and CTLA-4

The CD28 glycoprotein is expressed constitutively on the surface of 80% of human T cells (all $CD4^+$ cells and about 50% of $CD8^+$ cells express CD28) and on virtually 100% of murine T cells (5, 6). CD28 is also highly expressed on developing thymocytes, although its role in T cell development is not well understood (see below) (7, 8). CD28 expression is not static because the levels of CD28 increase on T cells following activation (9). However, ligation of CD28 with either anti-CD28 mAb or B7-1-transfected cells in the presence of PMA or PHA stimulation downregulates CD28 mRNA levels and cell surface expression. There was a concomitant reduction in CD28 signaling as CD28-induced calcium mobilization was significantly reduced (10). CTLA-4, on the other hand, is not constitutively expressed on T cells. Instead, it is upregulated following T cell activation due to positive regulatory elements (11–13; Z Wang, CE Donavan, H He, J Listman, G Guan, T Walunas, JA Bluestone, DL Perkins & PW Finn, unpublished observations). In both human (11) and murine (14) systems, cell surface expression of CTLA-4 peaks 48 h after activation, returning to background levels by 96 h. Thus, the period of time in which CD28 is transiently downregulated and less responsive to signaling is the time period

during which CTLA-4 expression is maximal, suggesting that CTLA-4 may be functionally active at a time when CD28 function is impaired.

The ligation of the CD28 molecule appears particularly important for CTLA-4 upregulation and function. $CD28^-$ human PBLs do not upregulate CTLA-4 mRNA following stimulation by PMA and calcium ionophore (15). Furthermore, anti-CD28 mAbs accelerate the kinetics of CTLA-4 mRNA accumulation among human PBL, such that CTLA-4 mRNA was observed as little as 1 h following activation in the presence of anti-CD28 and at a peak by 6 h (15). Finally, activated $CD28^{-/-}$ murine T cells did not express significant amounts of CTLA-4 at the cell surface unless exogenous IL-2 was added, suggesting that CD28 regulates CTLA-4 expression (14). Linsley and colleagues demonstrated a role for integrins in the regulation of CTLA-4 expression (11, 16). Purified human T cells do not upregulate CTLA-4 following stimulation with anti-TCR mAb alone. The addition of either an immobilized form of soluble ICAM-1, a ligand for LFA-1, or VCAM-1, a ligand for the VLA-4 accessory molecule, resulted in significant expression of CTLA-4 (11, 16). It is, therefore, likely that several signaling pathways play an important role in controlling the expression of CTLA-4.

Finally, one of the more interesting and puzzling aspects of CTLA-4 biology is the discordance between mRNA transcription and cell surface expression. We, and others, have shown that although mRNA levels are increased rapidly following T cell activation, cell surface expression is delayed (15). Activated $CD4^+$ and $CD8^+$ populations of both human (11, 13) and murine (12) origin express CTLA-4 mRNA, as do human and murine Th1 and Th2 T cell clones (13). Detailed studies have shown that CTLA-4 mRNA levels peak approximately 24 h following stimulation with PMA or alloantigen, subsequently decreasing to near background levels by 72 h (11, 15). In fact, in many T cell clones, little, if any, CTLA-4 is expressed following T cell activation even though mRNA is readily detectable in the same cells (R Abe, TL Walunas, C June & JA Bluestone, unpublished observations). In fact, efforts to develop full-length CTLA-4 transfectants and CTLA-4 transgenic mice have to date been unsuccessful. These results suggest that either CTLA-4 expression is post-transcriptionally regulated, or other, as yet unidentified, CTLA-4–associated proteins are needed for transport and cell surface expression.

General Protein Characteristics

Both CD28 and CTLA-4 exist as disulfide-linked homodimeric glycoproteins (4, 5, 11, 17). However, several groups have suggested that these molecules can also exist as monomeric proteins (15, 18). In our studies, murine CTLA-4 was found to exist on the surface of activated T cells as both a disulfide-linked dimer (with the monomer migrating between 33 and 37 kD) and a non–disulfide-linked

monomer (14). Individual anti-murine CTLA-4 mAbs preferentially precipitated monomeric vs dimeric forms of the glycoprotein (18). The two molecules are likely to exist in multiple isoforms on the cell surface (18). Discrepancies between the various studies may be the result of variations in the serological reagents. Both CD28 and CTLA-4 have an unpaired cysteine residue at a position just proximal to the transmembrane domain (19). This site is believed to be the site of dimerization for both CD28 and CTLA-4 monomers and could potentially be involved in the formation of heterodimers between CTLA-4 and CD28 glycoproteins. Although genetic and biochemical studies do not support this possibility, anti-human CTLA-4 mAb specifically immunoprecipitated CTLA-4 from COS cells cotransfected with human CD28 and CTLA-4 (11). In addition, there was no evidence for depletion of CTLA-4 following preclearing of membrane extracts of activated human T cells with anti-CD28 mAb (15). Finally, CTLA-4 precipitated from activated $CD28^{-}$-deficient ($CD28^{-/-}$) murine T cells was identical to that observed from $CD28^{+}$ T cells (14). Thus, it appears that heterodimerization of CD28 with CTLA-4 is not required for CTLA-4 expression on the cell surface, and vice versa, although low levels of functional heterodimer expressed on activated T cells cannot be completely ruled out.

Regulation of T Cell Activation by CD28 and CTLA-4

THE ROLE OF CD28 IN THE REGULATION OF CELL CYCLE PROGRESSION AND APOPTOSIS The proliferative responses of T cells isolated from $CD28^{-/-}$ mice or $CD28^{+}$ mice stimulated in the presence of CD28 antagonists are substantially reduced (21). However, recent studies have shown that CD28/B7-mediated signaling does not affect initial T cell proliferation (24–48 h) but attenuates the late proliferative responses (20, 21; JA Bluestone, unpublished observations). This is manifested as increased T cell death (apoptosis) late in culture that cannot be fully reversed by the addition of IL-2 or other survival cytokines. Thus, although CD28/B7 interactions clearly can facilitate the initiation of T cell responses, the major role of CD28 signaling may be to prevent apoptosis and help sustain proliferation. In this regard, a recent study has focused on the role of CD28 in the regulation of two genes believed to be involved in the prevention of cell death, bcl-2 and bcl-x. Both bcl-2 and the high molecular weight form of *bcl*-x, *bcl*-x_L, protect lymphocytes from apoptosis (22–24). Boise et al showed that while CD28 costimulation does not lead to any changes in the levels of bcl-2 found in activated T cells, that it does upregulate bcl-x_L and that this upregulation correlates with protection from cell death by a Fas dependent mechanism (25).

TH1/TH2 SUBSET DIFFERENTIATION CD28 engagement has been shown to enhance the production of various cytokines, including IL-1, IL-2, IL-4, IL-5,

TNF, and IFN-γ (5, 26, 27). However, recent studies in vitro and in vivo support a fundamental role for CD28 in the early development and differentiation of both Th1 and Th2 T cell subsets (28–30; LMC Webb, M Feldmann, personal communication). In the absence of CD28 signaling, naive T cells are biased toward a Th1 phenotype. Seder et al demonstrated that no IL-4 was produced when CD28/B7 interactions were blocked with hCTLA4Ig (28). In this system, the lack of IL-2 production was the underlying cause for the effect, since the addition of exogenous IL-2 overcame the defect in IL-4 production. In additional studies, differentiation of human Th1/Th2 subsets was shown to be dependent on CD28 ligation. Purified naive human T cells stimulated with anti-CD3 in the absence of CD28 costimulation produced only IL-2 and IFN-γ, whereas the addition of anti-CD28 mAbs induced both IL-4 and IL-5 (29; LMC Webb, M Feldmann, personal communication). The requirement for CD28 could not be overcome in these cultures by the addition of exogenous IL-4, suggesting that CD28 ligation may regulate other factors involved in T cell responsiveness to IL-4.

The importance of CD28 costimulation in the differentiation of Th2 cells is supported by several studies in vivo. The CD28$^{-/-}$ mice have a reduced Th2-dependent antibody response to VSV, while the Th1-dependent DTH response to LCMV remains intact (31). In addition, CD28$^{-/-}$ mice can reject allografts (DJ Lenschow, J Green, Y Zeng, CB Thompson, & JA Bluestone, unpublished data). The murine CTLA4Ig transgenic mice display a similar phenotype, with the most dramatic defect being a hyporesponsive B cell compartment (32, 33). T cells isolated from CTLA4Ig transgenic mice produce significantly decreased amounts of IL-4 and increased amounts of IFN-γ in response to primary immunization, and the B cells demonstrated impaired humoral responses to multiple antigens as compared to wild-type animals (32, 33). Furthermore, in the murine model of leishmaniasis the administration of hCTLA4Ig at the time of inoculation resulted in protection in the susceptible, Th2-type BALB/c mouse strain, but it had no effect in the resistant, Th1-type C57BL/6 mouse strain (34). Finally, hCTLA4Ig treatment blocked the induction in vivo of an IL-4 response to a nematode parasite, *Heligmosomoides polygyrus.* Mice treated with hCTLA4Ig at the time of infection generated significantly reduced numbers of IL-4 secreting cells, decreased levels of serum IgE, and reduced blood eosinophil counts (35). These results are most consistent with a model in which CD28-mediated signaling directly regulates Th1/Th2 differentiation.

CTLA-4 FUNCTION Although CD28 is clearly a costimulatory molecule, the function of CTLA-4 remains controversial. Early studies predicted that CD28 and CTLA-4 might play similar roles in the regulation of T cell responses, due to the amino acid sequence homology and ligand binding specificity. The first studies examining the role of CTLA-4 in proliferative responses in vitro

suggested that CTLA-4, like CD28, was indeed a costimulatory molecule. Immobilized anti-human CTLA-4 mAbs in the presence of TCR stimulation augment proliferation, although to a lesser degree than does anti-CD28-mediated costimulation. When both mAbs were present together with the anti-TCR stimulus, synergistic proliferation was observed (11). However, additional functional studies in both the murine and human systems suggested a different role for CTLA-4. Whereas anti-murine CTLA-4 mAb augmented proliferation of purified T cells, monovalent Fab fragments of the anti-CTLA-4 mAb, which do not cross-link CTLA-4, also augmented proliferation (14). Thus, CTLA-4 cross-linking may not transduce a positive signal, but rather the anti-CTLA-4 mAbs may block a negative signal by interrupting the interaction of CTLA-4 with its counterreceptors. In fact, whole anti-CTLA-4 mAbs inhibited anti-CD3-mediated T cell activation in the presence of optimal CD28 costimulation (14, 36). Recent studies in vivo also support the role for CTLA-4 as a downregulatory molecule in T cell activation. Kearney et al have described an adoptive transfer model in which antigen-specific transgenic T cells can be monitored for their expansion following exposure to antigen (37, 38). In these studies, animals treated with either anti-CTLA-4 mAb or Fab fragments of the anti-CTLA-4 mAb showed augmented antigen-mediated clonal expansion. There was also a delay in the decay in T cell numbers over time, suggesting that the anti-CTLA-4 mAb blocked programmed cell death in vivo (38). Thus, during T cell activation, CTLA-4 is upregulated, and through interactions with its ligands may facilitate or promote downregulation of the immune response. In this regard, Gribben et al have shown that ligation of CTLA-4 may mediate apoptosis (39). When preactivated human T cells were restimulated with antigen in the presence of an anti-CTLA-4 mAb, the proliferation was decreased, and the majority of T cells underwent programmed cell death. Induction of apoptosis in the presence of anti-CTLA-4 mAb could be blocked by coculturing the cells with either anti-CD28 mAb or exogenous IL-2, consistent with recent evidence that CD28 signaling upregulates the cell survival factor bcl-x_L (25). These results suggest that CTLA-4 may function through an association with an additional cell surface molecule.

THE B7-1 (B7/CD80) AND B7-2 (B70/CD86) GLYCOPROTEINS

B7-1, a B cell activation molecule first described in 1981 by Yokochi et al (40), was the first ligand to be identified for CD28, and later for CTLA-4 (41). In addition to its expression on activated B cells, B7-1 was also detected on a variety of APCs including dendritic cells, Langerhans cells, activated

monocytes, activated T cells, and a variety of tumor lines (Table 1). Both the human and murine B7-1 genes were cloned by Freeman et al (42, 43) and shown to be members of the immunoglobulin superfamily. The functional importance of the B7-1 molecule has been demonstrated in a number of studies of T cell activation. Both anti-CD3 and PMA-induced T cell proliferation was augmented by the addition of B7-1 transfectants. The proliferation was enhanced in a CD28-dependent fashion since T cell activation was blocked by anti-CD28 mAbs (44, 45). Furthermore, the potent costimulatory role of B7-1 has been demonstrated in vivo in transgenic mice in which B7-1 was ectopically expressed on the cells of the islets of Langerhans (46–48).

Despite the apparent ability of B7-1 to provide sufficient costimulation when expressed on transfectants or transgenic mice, it has been very difficult to demonstrate its function on normal antigen-presenting cells in mice. Anti-B7-1 mAbs minimally inhibit a primary mixed lymphocyte response, while hCTLA4Ig inhibits the response by as much as 80% (49). Additionally, the staining of either LPS-activated B cells or whole spleen with hCTLA4Ig was not inhibited by anti-B7-1 mAbs, suggesting that an additional CTLA-4 ligand existed (49–52). Finally, B7-1$^{-/-}$ mice are capable of mounting an immune response to nominal antigens and antigen-presenting cells isolated from these mice could be stained with labeled hCTLA4Ig (53). These observations led to the identification and eventual cloning of a second B7 family member, B7-2 (54, 55).

The human B7-2 gene was cloned by Freeman et al (55) and Azuma et al (54). The overall structure of B7-2 was found to be very similar to B7-1 with an extracellular domain containing two Ig-like domains, a transmembrane domain, followed by a cytoplasmic tail (54, 55). The cytoplasmic tail contains three potential sites for protein kinase C phosphorylation, indicating a potential signaling role for this molecule. In fact, the B7-2 molecule is phosphorylated following B cell activation (L Lanier, unpublished observations). The murine homolog of B7-2 has also been cloned (56, 57). These genes share only 25% amino acid homology with their B7-1 counterparts. However, several areas of homology, centered around sequences required for the formation of the Ig domains and potential CD28/CTLA-4 binding sites, have been reported (56).

Similar to B7-1 transfectants, B7-2 transfectants augment T cell proliferation and IL-2 production to suboptimal stimulation with anti-CD3 or PMA. This costimulation was inhibited by either hCTLA4Ig or anti-CD28 Fab, but not by anti-B7-1 mAbs, demonstrating therefore that B7-2 binds to both CD28 and CTLA-4 (54–56). While anti-B7-1 mAbs have been inefficient in their ability to block primary allogeneic MLRs, in most cases, the anti-B7-2 mAbs inhibited the responses to levels similar to hCTLA4Ig (58–60). A combination of both

Table 1 Cellular distribution of human and mouse B7-1 and B7-2

Cell type	Stimulation Conditions	hB7-1	hB7-2	mB7-1	mB7-2	References
B cell	Resting	none	none	none	low	(42, 49, 54, 58)
	Cultured	-[a]	↑	-	↑	(54, 58)
	LPS	↑	↑	↑	↑	(49, 54, 58, 60)
	Ig cross-linking[c]	↑	↑	-	↑	(43, 58, 59)
	Anti-CD40	↑	↑	↑	↑	(54)
	IL-2	↑	NR[b]	NR	↑	(71, 72)
	IL-4	↑	↑	↑	↑	(71, 72)
	IL-5	NR	NR	NR	↑	(58)
	IFN-γ	NR	NR	NR	↑	(72)
T cells	Resting	none	none	none	low	(54, 58, 63)
	Anti-CD3	↑	↑	↑	↑	(54, 58, 112)
T cell clones	Resting	-	-	-	-	(54, 112, 114)
	Anti-CD3	↑	↑	↑	↓	(54, 112, 114)
Peripheral blood monocytes	Resting	low	high	none	NR	(42, 54)
	IFN-γ	↑	↑	↑	NR	(42, 54, 68, 73)
	GM-CSF	↑	↑	NR	NR	(68)
	FcR cross-linking	↓	↓	NR	NR	(68)
Peritoneal macrophages[d]	Freshly isolated	NR	NR	low	low	(58)
	LPS	NR	NR	↑	↑	(58)
	IFN-γ	NR	NR	↓	↑	(58)
	IFN-γ + IL-10	NR	NT	↓[e]	↓[e]	(75, 115)
Peripheral blood dendritic cell	Resting	low	high	low	low	(54)
	IL-10	-	↓	NR	NR	[f]
Splenic dendritic cells	Freshly isolated	NR	NR	low	low	(63)
	Cultured	NR	NR	↑	↑	(49, 63, 116)
	LPS	NR	NR	NR	-	(63)
Langerhans cells	Freshly isolated	none[e]	low[e]	none	low	(63, 117)
	Cultured	↑	↑	↑	↑	(63, 117)

[a]No change
[b]NR = not reported
[c]The cross-linking of surface Ig with antigen or anti-Ig did not induce B7-1. B7-1 was induced when surface Ig was highly cross-linked by Ig-dextran.
[d]Peritonal macrophages were thioglycollate induced.
[e]These cells were stained with CTLA4Ig and therefore B7-1 and B7-2 cannot be differentiated.
[f]C Buelens, F Willems, A Delvaux, G Pierard, J-P Delville, T Velu & M Goldman, personal communication.

anti-B7-1 and anti-B7-2 mAbs were the most effective at inhibiting the MLR (61, 62). The role for both molecules in primary responses is further supported by the finding that a combination of anti-B7-1 and anti-B7-2 antibodies can induce anergy (61).

Resting B cells express no detectable B7-1 and very low levels of B7-2, while both B7-1 and B7-2 are upregulated following B cell activation with agents such as LPS, Con A, or cAMP (42, 49, 54, 58) (Table 1). However, dramatic differences exist in the kinetics and the signals that control B7-1 and B7-2 expression. The induction of B7-2 occurs within 6 h of stimulation, with maximal levels of expression achieved between 18 and 24 h (49, 58). In contrast, B7-1 expression is not detected until 24 h post stimulation and does not reach maximal levels until 48 to 72 h later (49, 58). Furthermore, activated B cells and dendritic cells expressed quantitatively higher levels of B7-2 than of B7-1, since hCTLA4Ig staining was almost completely inhibited by anti-B7-2 mAbs, while anti-B7-1 mAbs had little effect (49, 58, 63). Differences in expression were observed not only on B cells, but also on T cells. Freshly isolated human and murine T cells express low levels of B7-2 but not of B7-1 (54, 58, 63). Both B7-1 and B7-2 expression were upregulated following activation with anti-CD3 (54, 58). The regulation of B7-1 and B7-2 expression is controlled by cell-cell interactions and cytokines. Signals delivered through the cytoplasmic tail of the MHC class II molecules induced B7-1 expression on B cells (65, 66). Cells expressing tailless class II were profoundly deficient in their antigen presenting capacity, which correlated with a lack of B7-1 induction. Treatment of these cells with dibutyryl-cAMP restored their ability to present antigens by inducing B7-1 expression. The cross-linking of surface Ig also regulated B7-1 and B7-2 expression. B7-2 was rapidly induced on the B cell surface following cross-linking with anti-Ig or antigen (58, 59).

Following the engagement of antigen, the ability of surface Ig to rapidly induce functional B7-2 transforms a resting B cell into a fully competent antigen presenting cell (67). In contrast, BCR cross-linking with either anti-Ig or antigen did not induce detectable levels of B7-1, although IgD-coated beads resulted in some increased expression of B7-1 (43, 58). Together these results suggest that the degree of cross-linking determines whether or not B7-1 is upregulated. Interestingly, engagement of the Fc receptor downregulates both B7-1 and B7-2 on monocytes that have been activated with either IFNγ or GM-CSF (68). Thus, the engagement of distinct cell surface molecules may have antagonistic effects and may, in part, explain the inhibitory effect of Fc receptor cross-linking on B cell function. Finally, the CD40/CD40L pathway plays an important role in controlling B7-1 and B7-2 expression. Both B7-1 and B7-2 were induced by signaling through CD40, either through anti-CD40 mAbs or

activated T cells that expressed the CD40 ligand (69, 70). Thus, a variety of cell surface glycoproteins on B cells can regulate B7-1 and B7-2 expression and APC function.

An additional level of control of B7-1 and B7-2 expression results from the influence of cytokines. A number of cytokines have been shown to differentially regulate B7-1 and B7-2 expression. IL-4 is one of the most potent inducers of B7-2 and, to a lesser extent, B7-1 on B cells (71, 72). Incubation of small resting B cells with IL-4 upregulates B7-2 expression within 6 h with maximal induction occurring by 24 h (72). IFNγ increases the expression of B7-2 on B cells, peritoneal macrophages, and peripheral blood monocytes (58, 68, 72). IFNγ also increases the expression of B7-1 on peripheral blood monocytes but, surprisingly, downregulates expression of B7-1 on peritoneal macrophages (58, 73). IL-10 blocks both B7-1 and B7-2 upregulation on peritoneal macrophages and downregulates B7-2, but not B7-1, on human dendritic cells (74, 75; C Buelens, F Willems, A Delvaux, G Pierard, J-P Delville, T Velu & M Goldman, personal communication). These results suggest that the immunosuppressive properties of IL-10 may, in part, be a result of its regulation of CD28/CTLA-4 ligands. Thus, the differences in the ability of the respective cytokines to regulate the levels and temporal expression of B7-1 and B7-2 both qualitatively and quantitatively may result in distinct effects during an immune response.

ROLE OF CD28/B7 IN T CELL–B CELL INTERACTIONS

In order for B cells to enter the cell cycle, produce Ig, and undergo Ig class switching or somatic hypermutation, it is necessary for them to receive the appropriate T cell help (76). Recent work has demonstrated that both CD28/B7 and CD40/CD40L signaling pathways play a critical role in B cell responses. However, the interactions of the CD28/B7 and CD40L/CD40 pathways are not well understood. Early studies showed that signaling through the CD28 receptor can increase the surface expression of CD40L (77, 78; SJ Klaus, J Rosser, EA Clark, personal communication). Furthermore, the activation of the T cells by cross-linking CD28 with either antibody or with B7-1 transfectants enhanced the production of IgG and IgM by B cells in vitro as compared to anti-CD3-stimulated T cells alone (77). However, signaling through the TCR alone was sufficient for CD40L upregulation, and hCTLA4Ig did not block CD40L expression or the ability of these antigen-activated T cells to provide B cell help (70, 78). These results are consistent with the idea that CD28 ligation is not required for CD40L induction, but, as reported, CD28 ligation stabilizes CD40L mRNA, allowing for more rapid translation and transport of $CD40_L$ the T cell surface, and therefore results in increased B cell responses (78; SJ Klaus, J Rosser, EA Clark, personal communication).

The interrelationship between the CD28/B7 and CD40L/CD40 pathways also plays an important role in the ability of B cells to present antigen. While resting B cells express little if any B7-1 or B7-2, CD40 ligation induces their expression. B cells activated with anti-CD40 mAbs stimulated an allogeneic MLR more efficiently, and this stimulation was blocked by hCTLA4Ig. Further investigation revealed that CD40 cross-linking by mAbs, or the interaction of CD40 with its ligand CD40L, upregulated B7-1 and B7-2 (69). Incubation of resting B cells with activated T cells is another potent means of inducing B7-1 and B7-2 (70). In this system, B7-1 upregulation was inhibited by either anti-CD40 or by anti-CD40L mAbs consistent with the notion that CD40L/CD40 regulates B7-1 expression (70). On the other hand, the CD40L antagonist did not completely inhibit the ability of activated T cells to upregulate B7-2 (70). Therefore, other signals mediated by class II engagement or cytokines are involved in B7-2 regulation by activated T cells.

A TEMPORAL MODEL FOR THE REGULATION OF CD28/B7 FAMILY MEMBERS

Based on the studies summarized above, it is clear that the interactions of the CD28/B7 family members are highly regulated and quite complex. One model for the regulation of immune responses by CD28/B7 family is based on the kinetics of expression of the various CD28/B7 ligands and the ability of the B7-1, B7-2, CD28, and CTLA-4 molecules to cross-bind. First, B7-2 is rapidly induced on B cells through the engagement of surface Ig with antigen, cytokines produced by previously activated T cells, or CD40 cross-linking by activated T cells. Next, antigen-specific naive or activated $CD28^+$ T cells can interact with the $B7\text{-}2^+$ B cells expressing a complete MHC/antigen complex. The simultaneous engagement of the TCR and the CD28 molecules results in the activation of the T cell and prevents the induction of anergy. Signals generated through the T cell receptor induce the expression of CD40L on naive cells, which is further stabilized by CD28 signals. The upregulation of CD40L then allows the T cell to provide the necessary B cell help for the production of antibodies. Concurrently, APCs upregulate B7-1 levels, via signaling through MHC class II, CD40, and perhaps lymphokines secreted by the activated T cells.

One to three days after this cellular interaction occurs, CTLA-4 is upregulated on T cells. Several interactions are possible at this time since the B cells express both B7-1 and B7-2, while the T cells express both CD28 and CTLA-4. Since CTLA-4 has a higher affinity for B7-1 and B7-2 than does CD28, these costimulatory ligands may preferentially interact with CTLA-4 on the activated T cells to downregulate the immune response either by directly inducing

apoptosis or by competing with CD28 for its ligands and preventing the upregulation of protective factors such as bcl-x_L. Thus, under normal circumstances, the CD28/B7 system will serve to both upregulate and downregulate immune responses. Any disturbance in the CD28/B7 system may disregulate the immune response and affect the development and progression of the immune response.

IN VIVO REGULATION OF IMMUNE RESPONSE BY MANIPULATION OF THE CD28/B7 PATHWAY

Activation of Immune Responses

Both B7-1 and B7-2 costimulate T cells through their interaction with CD28. The expression of one of these ligands on a cell that lacks costimulatory molecules could convert a nonfunctional APC into a functional one. This may have important implications in upregulation of immune responses. In fact, CD28/B7-mediated costimulation has provided a new approach to cancer therapy, because the inability of some tumors to induce immune responses has, in some cases, been correlated with a deficiency in providing costimulatory signals. The introduction of B7-1 or B7-2 into tumor cells in several models enhances the anti-tumor response (79–86). In fact, in some studies, the immunization of mice with the B7-1-expressing tumor protected the animal from further challenge with nontransfected parental tumors (80–86). However, the transfection of B7-1 into tumor cells has not been uniformly successful in inducing tumor immunity. In one instance, a potent immune response was induced and the B7-1 transfected tumors were rejected, but these same tumors could not protect from further challenge with the parental tumor (79). Furthermore, the lack of an anti-tumor response did not always depend on a deficient costimulatory response. Several relatively nonimmunogenic tumors did not induce an immune response, even when cotransfected with both B7-1 and B7-2 (83, 85, 87). These results suggest that certain tumors may be lacking additional cell surface molecules needed for complete T cell activation. These additional factors may include the tumor antigen itself, MHC molecules, or other cell surface costimulators. In support of this possibility, cotransfection of one of the nonimmunogenic tumors with both B7-1 and ICAM-1 resulted in its immune recognition and rejection (86).

Studies have begun to compare the relative costimulatory activity of B7-1 and B7-2. The majority of studies in vitro and some tumor studies in vivo (87) suggested that B7-2 was as effective a costimulatory molecule as B7-1, recent experiments in which either B7-1 or B7-2 were transfected into tumors have suggested that B7-1 is a more potent costimulator in some tumor models (CJ Bartels, JC Yang, personal communication; T Gajewski, personal

communication). In another study, in which the animals were immunized with irradiated tumor cells, only the B7-1-transfected tumors were able to generate tumor-specific CTLs and subsequently to protect against tumor challenge (T Gajewski, personal communication). Interestingly, in both models, the combined expression of B7-1 and B7-2 on the tumor cells was less effective in stimulating an anti-tumor response than was expression of B7-1 alone. This may reflect a competition between B7-1 and B7-2 for binding to CD28. Alternatively, the addition of B7-2 to the transfectant may hyperstimulate the immune response to shut down T cells either directly, perhaps through its interaction with CTLA-4, or by increasing the potency of the T cell signals to promote Th2 responses that inhibit tumor immunity (see below).

Suppression of Immune Responses

In vitro studies have demonstrated that the CD28/B7 pathway is critical in T cell activation. The interruption of this pathway leads to an inhibition of T cell proliferation and, under some circumstances, induces either antigen-specific hyporesponsiveness or anergy (88, 89). Therefore, targeting this pathway in vivo may represent a novel method of immunosuppression in which only the antigen-specific T cells would be tolerized. To examine this possibility, several investigators have utilized hCTLA4Ig to interrupt the CD28 signaling pathway in several models including transplantation, autoimmune disease, antibody responses, and parasite challenge.

TRANSPLANTATION The importance of CD28/B7 interactions in vivo was first established in the transplant setting. CTLA4Ig treatment effectively prolonged graft survival and, in many cases, induced donor-specific tolerance. As an example, in a xenogeneic islet transplant model, diabetic mice transplanted with human islets and treated with CTLA4Ig exhibited long-term survival of the xenogeneic islets (90). Furthermore, this short treatment induced donor-specific tolerance. hCTLA4Ig-treated mice that were retransplanted with either donor or third party islets rejected only the third party islets. Human CTLA4Ig prolonged graft survival in both allogeneic rat cardiac and murine islet transplant models, but eventually all of the grafts were rejected (62, 91). Human CTLA4Ig treatment also reduced the lethality of allogeneic graft-vs-host disease (GVHD) across an MHC barrier but did not alleviate all of the symptoms of GVHD (92, 93). These results suggest that allogeneic transplantation may be more difficult to manipulate than the xenogeneic systems. However, recent efforts to augment the effectiveness of hCTLA4Ig have been successful. Pearson et al found that hCTLA4Ig treatment could induce donor-specific tolerance in a rat cardiac allograft model in certain strain combinations (94). In the studies performed by Turka and colleagues, changes in the dose regimen or the addition

of donor-specific transfusions led to donor-specific tolerance (95, 96). Finally, the addition of other immunosuppressive drugs maximizes the hCTLA4Ig-mediated suppression. The blockade of CD28/B7 and LFA-1/ICAM-1 interactions was more effective than hCTLA4Ig alone at preventing GVH disease-induced lethality (BR Blazar, PA Taylor, A Panoskaltsis-Mortari, GS Gray, DA Vallera, personal communication). Treatment of mice with hCTLA4Ig and suboptimal doses of cyclosporin A (CsA) at the time of transplant resulted in graft survival beyond that of hCTLA4Ig alone and in some cases resulted in indefinite graft survival (97; Y Zeng, JA Bluestone, unpublished observations). The ability of hCTLA4Ig and CsA more effectively to block transplant rejection has important clinical implications because current transplant therapies utilize CsA. All in all, these studies demonstrated the effectiveness of interrupting the CD28/B7 signaling pathway in promoting transplant tolerance. These studies also suggest that the timing of the treatment or the strength of TCR signal may be important in determining the efficacy of hCTLA4Ig immunosuppression.

HUMORAL RESPONSES One immune response that has been profoundly inhibited by hCTLA4Ig therapy is the humoral immune response. Primary antibody responses to soluble proteins such as KLH or cell-bound antigens such as sheep red blood cells were inhibited by hCTLA4Ig in a dose-dependent fashion (98). Treatment with hCTLA4Ig could be delayed as long as 3 days following antigen priming and still suppress in vivo antibody production. Human CTLA4Ig therapy during the primary response also reduced secondary and tertiary responses to the nominal antigen. The amount of reduction correlated with the concentration of hCTLA4Ig used in the initial treatment, suggesting that the immunosuppression lasted only for the duration of circulating serum levels (98). However, anti-B7-2 mAb treatment not only blocked antibody production to antigenic challenge but also suppressed somatic hypermutation and had long-term effects on B cell memory responses (99). Therefore, the use of the more potent CD28 antagonists may be the most promising treatment in vivo.

Further evidence that CD28/B7 signaling plays an important role in antibody responses has come from the examination of $CD28^{-/-}$ mice. Antibody production in these mice was severely impaired. Basal immunoglobulin levels are only 20% of that observed in normal mice. Furthermore, the relative proportions of the different IgG subclasses were altered. The serum of these animals contains dramatically decreased levels of IgG1 and IgG2b, while the levels of IgG2a were increased as compared to normal mice. B cell responsiveness in the $CD28^{-/-}$ mice appeared to be intact since normal levels of anti-VSV Abs of the IgM class (a T-independent response) were produced in response to a primary challenge (31). However, the T cell help necessary for antibody class switching

following a second challenge was not intact in these mice. Transgenic mice expressing soluble murine CTLA4Ig displayed a phenotype very similar to that of $CD28^{-/-}$ mice. T-independent antibody responses were intact, but immune responses to T-dependent antigens were severely impaired (32, 33). Primary responses to DNP-KLH were eliminated. Only after two and three stimulations could reduced levels of antibodies be detected, and these were of the IgM, not the IgG isotype. This deficiency in T cell help was also evident by a lack of germinal center formation, isotype switching, and somatic hypermutation (32, 33) similar to what had been observed in the anti-B7-2–treated animals (99). Therefore, blockade of the CD28/B7 interactions, either from the CD28 side or the B7 side, results in a profound defect in the ability of B cells to respond to antigen in vivo.

AUTOIMMUNE DISEASES CTLA4Ig therapy has also had significant effects on the clinical course of several autoimmune diseases. We have shown that blockade of the CD28/B7 pathway has profound effects on the development of diabetes in the NOD mouse model. In these studies, hCTLA4Ig treatment of young NOD mice inhibited the onset of diabetes that occurred 6 to 18 weeks after treatment was stopped and the protein had been cleared from the serum (100). In a relapsing model of experimental autoimmune encephalomyelitis (R-EAE), hCTLA4Ig treatment during antigen priming blocked the development of clinical disease (101). This was also true in a T cell adoptive transfer model of R-EAE (102). In the murine model for systemic lupus erythematosus (SLE), NZB/NZW F_1 mice (B/W) spontaneously develop a lupus-like autoimmune disease characterized by the production of autoantibodies to self-molecules such as dsDNA (103). The treatment of these mice with murine CTLA4Ig prior to the detection of autoantibodies significantly inhibited autoantibody production and disease progression, even after cessation of treatment (104). Thus, blockade of CD28/B7 signaling can control early events involved in the induction of several different autoimmune diseases. However, most autoimmune diseases are diagnosed after initial responses to the autoantigen(s). Therefore, it was important to examine the effectiveness of these therapies on established disease. Treatment of the lupus-prone mice with murine CTLA4Ig during the late stages of the disease effectively intervened in disease progression and prevented the production of further autoantibodies (103). In addition, NOD mice treated with hCTLA4Ig after the onset of insulitis also had a reduced incidence of diabetes (100). Finally, relapses of clinical disease in R-EAE were inhibited by F(ab) fragments of anti-B7-1 mAbs when treatment was initiated after the resolution of the acute phase of disease (SD Miller, CL Vanderlugt, DJ Lenschow, MC Dal Canto, JA Bluestone, unpublished observations). Together, these data demonstrate an important role for CD28/B7 signaling in both the initiation and propagation of several autoimmune diseases.

DIFFERENTIAL EFFECTS OF B7-1 AND B7-2 LIGATION

It is becoming increasing clear that the B7-1 and B7-2 molecules may differentially control the immune response as a consequence of one or more of the distinctive properties of these costimulatory ligands. For instance, the expression of B7-1 and B7-2 varies on B cells, T cells, macrophages, and dendritic cells depending on the activation state of the cells (Table 1). An additional level of complexity involves the ability of cytokines to either induce or suppress the expression of these costimulatory ligands. Therefore, either B7-1 or B7-2 may dominate during different stages of the immune response. Furthermore, there are increasing data to suggest that these two molecules do not bind the CD28 and CTLA-4 molecules similarly (105). Both B7-1 and B7-2 bind to hCTLA4Ig with a 20–100-fold higher avidity than they do to CD28Ig. In addition, Linsley and colleagues showed that B7-1 has a slightly higher avidity for CD28 and CTLA-4 than does B7-2. More significantly, B7-2 dissociates more rapidly from hCTLA4Ig than does B7-1, and hCTLA4Ig was less effective at inhibiting B7-2–dependent responses (105). Finally, the fine specificity of the B7-1 and B7-2 interaction with hCTLA4Ig is different. Previous studies have shown that the region of CTLA-4 and CD28 that is the site of interaction with both B7-1 and B7-2 is a transmembrane proximal hexapeptide, MYPPPY, found in the region of greatest sequence homology between CD28 and CTLA-4. A hCTLA4Ig construct containing a single amino acid mutation in the MYPPPY motif showed reduced but still significant binding to B7-1. However, binding to B7-2 was completely abolished. Thus, amino acids within the CDR1 and CDR3 regions of the CTLA-4 molecule are responsible for the enhanced avidity of CTLA-4 for B7-1 (106). However, the precise interaction sites for B7-1 and B7-2 with CD28 and CTLA-4 may be distinct (105).

Recent studies, in vitro and in vivo, suggest that these differences may directly effect CD28-mediated costimulation. Kuchroo et al demonstrated that T cells from a myelin basic protein-specific TCR transgenic mouse secreted predominantly IFN-γ when cultured in the presence of anti-B7-2 mAbs, while cultures carried out in the presence of anti-B7-1 mAbs led to increased IL-4 secretion (107). Studies by Freeman et al also support an important role for B7-2 in the signaling of IL-4 production (108). While transfectants of B7-1 or B7-2 induced similar levels of IL-2 and IFN-γ, only B7-2 transfectants were able to induce IL-4. Continuous restimulation by the B7-2 transfectants resulted in the induction of greater amounts of IL-4 (108). Together, these results suggest that B7-1 and B7-2 may directly control Th1 vs Th2 development, respectively. In vivo studies also suggest that the B7-1 and B7-2 costimulatory ligands play distinct roles during the initiation and propagation of an immune response. Kuchroo and colleagues found that treatment with anti-B7-1 mAbs

during R-EAE induction protected mice from disease, while anti-B7-2 mAbs exacerbated disease severity (107). In this model, the investigators correlated the presence of a Th2 response with the protective therapy and suggested that the anti-B7-2 mAb therapy skewed the T cell response toward a Th1 phenotype. However, a number of other studies in vitro and in vivo have shown that B7-1 and B7-2 regulate both Th1- and Th2-mediated responses. First, two studies have shown that both B7-1 and B7-2 transfectants can provide costimulatory signals for both Th1 and Th2 lymphokine production (109, 110). Second, the primary immune response appears to be most dependent on B7-2 since B7-1$^{-/-}$ mice have relatively normal Th1- and Th2-dependent responses, while B7-2$^{-/-}$ mice are severely compromised (A Sharpe, personal communication). Furthermore, in an allogeneic islet transplant model, anti-B7-2 but not anti-B7-1 mAbs prevented graft rejection. The combination of the two mAbs was most effective in prolonging allograft survival (62). Since graft rejection has been suggested to be Th1-mediated, B7-1 rather than B7-2 would have been expected to be dominant in this setting. Furthermore, the treatment of mice with anti-B7-2 mAbs during priming in vivo with antigen (a Th2-dependent immune response) inhibited the development of antibody responses (52, 99). Anti-B7-2 mAb treatment was also able to block disease progression in the NOD model for autoimmune diabetes (100). Thus, it appeared as if B7-2 played a dominant role as the costimulatory ligand for CD28 in transplantation, humoral responses, and initiation of autoimmune disease.

More recent studies have shown that B7-1 can regulate immune responses, especially following initial antigen exposure. Treatment of NOD mice with anti-B7-1 mAbs accelerated the disease course and exacerbated the inflammatory response in female mice treated at 2 to 4 weeks of age (100). Furthermore, anti-B7-1 treatment of normally resistant male mice developed disease at a rate and frequency similar to that observed in untreated female NOD mice (100). A similar exacerbation of disease was observed with anti-B7-1 mAbs in a murine R-EAE model (SD Miller, CL Vanderlugt, DJ Lenschow, MC Dal Canto, & JA Bluestone, unpublished observations). Treatment initiated after resolution of the acute phase of disease resulted in an increased rate of onset, frequency, and severity of the relapses. However, treatment with non-cross-linking F(ab) fragments of the anti-B7-1 mAbs immediately after the resolution of the acute phase of disease blocked both clinical relapses and epitope spreading in R-EAE. Two points can be made from these studies. First, the exacerbation caused by the intact anti-B7-1 mAb may be due to its direct signaling of either the APCs or activated T cells. This possibility has important implications for all the in vivo studies that utilize antibody therapy. Second, there appear to be distinct differences on the outcome of the mAb therapy depending on the temporal

administration of the antibodies. The B7-2 molecule appears to play a critical role prior to antigen exposure (transplant setting and humoral responses), whereas B7-1 plays an important role in the control of the immune response after antigen exposure. One explanation for these differences may be our recent observation that B7-1 expression is preferentially upregulated during the acute phase of an autoimmune response (SD Miller, CL Vanderlugt, DJ Lenschow, MC Dal Canto, & JA Bluestone, unpublished observations). In fact, many of the in vivo effects of the CD28 antagonists can be explained by the timing of antibody therapy. The treatment of BALB/c mice with hCTLA4Ig during infection with *L. major* protected the normally susceptible strain from disease, by decreasing the production of IL-4, while the mice were no longer protected from disease by hCTLA4Ig if treatment was delayed by more than one week post-infection (34). The efficiency of hCTLA4Ig blockade of antibody responses was also decreased as treatment was delayed (98). Finally, in an allogeneic cardiac transplant model, delaying treatment with hCTLA4Ig by 2 to 3 days post-transplant resulted in long-term survival characterized by an inhibition of Th1 but not Th2 cytokines (96). Thus, a variety of factors, including the nature of the APC expressing the B7 molecules, the affinity of the B7 molecules for CD28 or CTLA-4, and the level of B7-1 and B7-2 expression, may determine the relative roles played by each ligand during an immune response. Furthermore, the timing of treatment with CD28 antagonists can influence the Th1 and Th2 subset development.

STRENGTH OF SIGNAL MODEL

Based on all of these observations, we propose a model of costimulation that integrates many of the observations related to TCR ligation, CD28 costimulation, and Th1/Th2 biology (Figure 1). The "strength-of-signal" hypothesis suggests that the intensity of T cell signaling not only determines the potential to initiate a response but can dramatically affect the balance of Th1/Th2 subsets. We speculate that CD28 costimulation can have distinct effects on the immune response depending on antigen dose, APC function, cytokine milieu, and level of costimulation. Under conditions of low antigen density, CD28 ligation is essential since anti-B7 mAbs and hCTLA4Ig therapy or genetic disruption of the CD28 molecule results in a diminished ability to generate a productive primary T cell response and severely suppresses T-dependent humoral responses. Under these, perhaps physiological, conditions, stimulation of primary T cell responses is largely dependent on B7-2-mediated costimulation. At early time points in the immune response, B7-2 is expressed constitutively on dendritic cells and functions to regulate both Th1 and Th2 responses. As the immune response progresses, both B7-1 and B7-2 are upregulated, resulting in increased

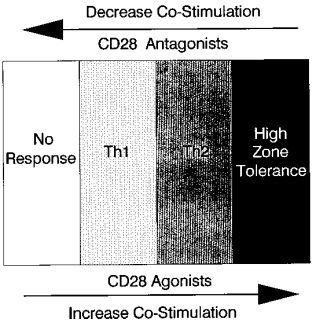

Figure 1 The strength-of-signal model.

costimulatory signals and an increased strength-of-signal that promotes T cell expansion and cytokine production and may skew the T cell response toward the Th2 phenotype. Thus, any reagent or situation that reduces costimulation during this response reduces the strength-of-signal, promoting Th1 responses. For example, the treatment of BALB/c mice with hCTLA4Ig during infection with *L. major* protected the normally susceptible strain from disease by decreasing the amount of IL-4 being produced (34). Blockade of CD28 signaling also inhibited the production of an IL-4 response to *H. polygyrus* and decreased humoral responses (35, 52, 98, 99). Finally, the effect of anti-B7-2 mAbs observed by Kuchroo et al may reflect the blockade of initial costimulation during the induction of R-EAE, thus skewing the response to the disease promoting Th1 phenotype (107).

Finally, high levels of costimulation coupled with high TCR occupancy may, in fact, downregulate immune responses. This effect may occur due to the extensive signaling via the TCR and CD28, as has been suggested as an explanation for "high zone tolerance" or "clonal exhaustion." Alternatively, hyperstimulation may substantially upregulate CTLA-4 on the activated T cells. Since the ligation of CTLA-4 inhibits immune responses, the interactions of CTLA-4 with either B7-1 or B7-2 may further amplify the suppression observed under these conditions. In this regard, in some tumor models the expression of both B7-1 and B7-2 on a tumor is less effective at inducing an immune response than is a tumor on which is expressed one or the other (T Gajewski, personal communication; CJ Bartels, & JC Yang, personal communication). Furthermore, transgenic mice expressing high levels of B7-1 on their B cells are profoundly deficient in their ability to receive T cell help for antibody production (111). Therefore, the careful manipulation of the CD28/B7 signaling pathway may dramatically impact the course of an immune response.

CONCLUSION

The studies summarized in this review demonstrate the importance of the CD28/B7 signaling pathway and also begin to illustrate its complexities. First, CD28 functions by costimulating T cells and preventing the induction of either anergy or apoptosis. Second, a CD28 homologue, CTLA-4, counterbalances CD28 by downregulating the immune response either by competing with CD28 for its ligands or by inducing apoptosis. Third, two distinct molecules B7-1 and B7-2 function as costimulatory ligands for CD28 and CTLA-4. While B7-2 dominates in primary responses, the roles of B7-1 and B7-2 during an ongoing response depend upon: the relative expression of CD28/CTLA-4; the nature and concentration of cytokines in the surrounding milieu; and the characteristics of the APC that are encountered. Finally, manipulation of the CD28/B7 pathway can alter the balance of the immune response by influencing the nature of the cytokines produced in response to antigen exposure. A better understanding of the consequences of regulation of the CD28/B7 costimulatory interactions will offer new insights into important elements controlling the immune response and allow for the development of novel immunotherapies for cancer, transplantation, and autoimmune disease.

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T CELL ANTIGEN RECEPTOR SIGNAL TRANSDUCTION PATHWAYS

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ABSTRACT

The T cell antigen receptor (TCR) regulates the activation and growth of T lymphocytes. The initial membrane proximal event triggered by the TCR is activation of protein tyrosine kinases with the resultant phosphorylation of cellular proteins. This biochemical response couples the TCR to a divergent array of signal transduction molecules including enzymes that regulate lipid metabolism, GTP binding proteins, serine/threonine kinases, and adapter molecules. The ultimate aim of studies of intracellular signaling mechanisms is to understand the functional consequences of a particular biochemical event for receptor function. The control of cytokine gene expression is one of the mechanisms that allows the TCR to control immune responses. Accordingly, one object of the present review is to discuss the role of the different TCR signal transduction pathways in linking the TCR to nuclear targets: the transcription factors that control the expression of cytokine genes.

INTRODUCTION

T lymphocyte activation is controlled by the T cell antigen receptor (TCR) in combination with additional signals triggered by accessory molecules present on the surface of the antigen-presenting cells (APC). The T cell antigen receptor (TCR)/CD3 complex comprises the polymorphic TCR α and β subunits in a noncovalent association with the invariant chains of the TCR complex: the

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TCR ζ chains and the γ, δ, and ϵ chains of the CD3 antigen complex (1). The capacity of the TCR to transduce signals across the T cell membrane is mediated by the cytoplasmic domains of the subunits of the CD3 antigen and the ζ chains (2). The intracellular tails of the CD3 and ζ molecules contain a common motif, $EX_2YX_2L/IX_7YX_2L/I$, termed an immunoglobulin receptor family tyrosine-based activation motif (ITAM). This motif is crucial for TCR coupling to intracellular tyrosine kinases and hence absolutely required for all subsequent TCR signaling responses (3). The ITAMS are also found in the invariant subunits of the B cell antigen receptor and in the γ subunit of the FcϵRI and FcγRIIIA (4–6) where they play a similar role in antigen receptor signal transduction (3).

The earliest biochemical response elicited by the TCR is the activation of a protein tyrosine kinase (PTK) (7, 8). Two PTKs associate with the TCR/CD3 complex: the src family kinase p59fyn and a 70-kD PTK termed ZAP-70 and a second src family PTK p56lck are also critical for TCR function (2). The proposed sequence of events in the TCR-regulated PTK cascade is activation of src kinases, the phosphorylation of ITAMs followed by the recruitment, tyrosine phosphorylation, and activation of ZAP-70 (9, 10). The association of ZAP-70 with the TCR complex is mediated by SH2 domain interactions with ITAMS. ZAP-70 has two SH2 domains and binds preferentially to a doubly phosphorylated ITAM; this explains why both tyrosines within the ITAM are required for full ITAM function (8, 11).

TCR REGULATION OF INOSITOL LIPID METABOLISM

There has been considerable progress in the identification of the cellular substrates for the TCR-regulated PTKs. These include the TCR subunits (7) and other receptor molecules such as CD5 (12), cytoskeletal proteins like Ezrin (13), ZAP-70, phospholipase γ1 (2), VCP (vasolin containing protein) (14), the protooncogenes Vav (15), c-cbl (16), Shc (17). However, the first PTK-controlled TCR signaling pathway to be well characterized was that mediated by phospholipase Cγ1 (PLCγ1) (Figure 1). PLCγ1 regulates the hydrolysis of inositol phospholipids and thereby generates inositol polyphosphates and diacylglycerols, which allows the TCR to regulate intracellular calcium levels and the serine/threonine kinase family of protein kinase C isozymes (PKCs). PLCγ1 is tyrosine phosphorylated in TCR-activated cells, a process which is important for its activation (2). The TCR also induces the formation of a protein complex between the SH2 domains of PLCγ1 and a 36-kDa TCR-induced tyrosine phosphoprotein (18). p36 is a membrane-associated protein, and the interaction between p36 and PLCγ1 may be important in the recruitment of PLCγ1 to the plasma membrane—a necessary step because the substances

that are substrate for PLCγ1, the inositol phospholipids, are in the plasma membrane.

Another signaling pathway involving inositol lipid metabolism originates from the TCR; one controlled by a phosphatidylinositol 3′-hydroxyl kinase (PtdIns 3-kinase) (19) (Figure 1). PtdIns 3–kinase catalyses the phosphorylation of phosphoinositides at the D-3 hydroxyl of the myo inositol ring generating polyphosphoinositides PtdIns (3) P, PtdIns (3, 4) P_2, and PtdIns (3, 4, 5) P_3. PtdIns 3–kinase comprises a regulatory 85-kDa subunit that contains two SH2 domains and at its N-terminus, one SH3 domain and a catalytic 110-kDa subunit. Interactions between CD28 and its ligands B7-1 and B7-2 are considered to generate the crucial costimulatory signals for T cell activation (20, 21). The stimulatory effect of the TCR alone on PtdIns 3–kinase activity is small, and the combined triggering of the TCR and the costimulatory receptor CD28 are necessary for optimal activation of the enzyme (22). Nevertheless, a functional coupling between the TCR and PtdIns 3–kinase is demonstrated by the observations that the p110 PtdIns 3–kinase catalytic subunit is hyperphosphorylated on serine residues in response to TCR activation (23).

CD28 regulation of the PtdIns 3–kinase appears to involve recruitment of the enzyme to the membrane via an association between the SH2 domains of p85 and the tyrosine phosphorylated cytoplasmic domain of CD28 (24, 25). In addition, p85 binds to the tyrosine phosphorylated TCR ζ chain, but this association has not been consistently observed to correlate with PtdIns 3–kinase stimulation, indicating that it is not obligatory for activation of the enzyme

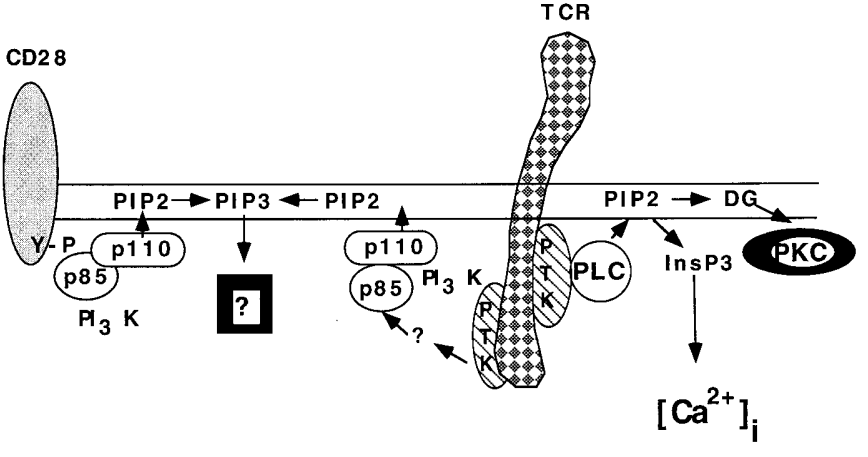

Figure 1

(26–28). Another possible mechanism to link the TCR to PtdIns 3–kinase involves src family kinases associated with the TCR complex that can bind to PtdIns 3–kinase via binding of their SH3 domains to two proline rich motifs in the p85 subunit of the enzyme (29, 30). However, attempts to find direct interactions between PtdIns 3–kinase and TCR-activated PTKs have yielded discrepant results depending on the T cell population examined (23). Nevertheless, the idea that SH3-mediated interactions may be important for the TCR/PtdIns 3–kinase link is an attractive concept.

The direct downstream targets of phosphoinositides phosphorylated at the D-3 position are not really known, but the use of inhibitors of the PtdIns 3–kinase has suggested that this enzyme is important in the regulation of the p70S6 kinase, a protein kinase thought to play an essential regulatory role in protein synthesis (31, 32). Further clues as to the role of PtdIns 3–kinases have been generated by experiments demonstrating that there are serine kinases whose in vitro activity is modified by PI_3P or PIP_3. These include members of the PKC family, particularly PKCs ϵ and ζ, which can be activated in vitro by PIP_3 (33). The PH domain of the serine threonine kinase Akt can bind PI_3P, resulting in its activation (34). PH domains are found in a large number of signal transduction molecules, including tyrosine kinases such as BTK and ITK (35). The hypothesis that PH domains are the signaling targets for the D-3 phosphoinositides is thus intriguing because it would mean that PtdIns 3–kinase could play a role in coupling cell surface receptors to quite diverse signal transduction pathways.

TCR REGULATION OF THE GTPASE RAS

The TCR is able to regulate the activation of the guanine nucleotide binding proteins p21ras via a PTK-dependent mechanism independent of TCR coupling to PLCγ1 (36). The level of active p21ras-GTP complexes is determined by a balance of the rate of hydrolysis of bound GTP and the rate of exchange of bound GDP for cytosolic GTP. The GTPase activity of $p21^{ras}$ is controlled by GTPase activating proteins such as p120-GAP and neurofibromin (37). The inhibition of Ras GAP proteins is almost certainly one mechanism used by the TCR to regulate the Ras guanine nucleotide binding cycle (36). However, recent biochemical studies have suggested that the TCR may also regulate $p21^{ras}$ via control of guanine nucleotide exchange proteins. In particular, many receptors in a variety of cells regulate $p21^{ras}$ by stimulating the guanine nucleotide exchange protein, Sos (reviewed in 38, 39). Sos is associated with an adapter protein Grb2 which is a 26-kDa polypeptide composed of one SH2 domain and two SH3 domains. The SH3 domains of Grb2 bind to the carboxy terminal proline-rich domain of Sos, and the Grb-2 SH2

domain binds to tyrosine phosphorylated molecules (38). Grb-2 has been implicated in regulation of $p21^{ras}$ in T cells because the TCR induces tyrosine phosphorylation of a 36-kDa protein that is found in a complex with the SH2 domains of Grb2 in TCR-activated cells (18, 40). Moreover, the formation of p36/Grb-2/Sos complexes is a rapid response to TCR ligation that correlates well with $p21^{ras}$ regulation (41). In many growth factor–stimulated cells, e.g. in T cells stimulated with the cytokine IL-2, there is tyrosine phosphorylation of an adapter protein Shc and rapid formation of Shc/Grb-2/Sos complexes (42). T cell activation via the TCR certainly results in tyrosine phosphorylation of Shc, but reports disagree as to whether Shc/Grb2/Sos complexes form. For example, Shc/Grb2/Sos complexes were detected in TCR-activated murine T cell hybridomas but not in TCR-activated peripheral blood–derived T cells (17, 43). Consideration of the discrepancies in the reported TCR-induced Grb2 complexes illustrates how there may be considerable heterogeneity of TCR responses in different cells types. It would be predicted for example that differences in the relative levels of Grb2/Shc/p36 in different populations of T cells would change the pattern of Grb2 interactions induced by the TCR.

The regulation of Grb2 protein complexes in T cells has been extensively analyzed at the biochemical level, but there is absolutely no data concerning the function of Grb2 in T lymphocytes. Moreover, T cells express another protein that can catalyze guanine nucleotide exchange on $p21^{ras}$: a molecule called C3G. C3G binds to the SH3 domains of an adapter molecule Crk. Crk has three SH3 domains and a single SH2 domain. TCR triggering results in tyrosine phosphorylation of Crk binding proteins analogous to the TCR regulation of Grb2 complexes (44). The relative contribution of Grb-2/Sos complexes and Crk/C3G complexes to $p21^{ras}$ activation in T cells is not clear, but any analysis of Ras regulatory mechanisms in T cells must explain PKC regulation of $p21^{ras}$ since this appears to be a lymphocyte-specific response. It is conceivable that there is a PKC-sensitive, lymphocyte-specfic Ras exchange protein, and one candidate for this molecule is the proto-oncogene Vav, which is expressed only in hematopoietic cells (45). However, Vav cannot explain the apparent lymphoid specificity of the PKC-$p21^{ras}$ link because Vav is expressed in all hematopoietic cells, and mast cells or myeloid cells that do express Vav do not express a PKC-mediated route for $p21^{ras}$ regulation. Moreover, the role of Vav as a Ras guanine nucleotide exchange protein is controversial, and structurally Vav is a more likely candidate as an exchange protein for the Ras-related *rho/rac* proteins (46). Nevertheless, Vav expression is apparently essential for antigen receptor function in both B and T cells, and its precise role in lymphocytes will be interesting to resolve (47–49).

THE FUNCTION OF CALCIUM, PKC, AND RAS IN ACTIVATION OF T CELL

The function of calcium, $p21^{ras}$, and PKC signals in TCR signaling has been explored mainly in the context of TCR regulation of the production of a T cell growth factor IL-2. It has been possible to explore the role of calcium and PKC in T cells by examining the consequences of stimulating T cells with calcium ionophores that elevate intracellular calcium and phorbol esters that activate PKC. The results of such experiments showed that the combination of these two pharmacological activators could substitute for TCR triggering in induction of the IL-2 gene, a fact prompting the conclusion that TCR control of calcium and PKC was sufficient to explain TCR control of T cell activation. However, it is now recognized that in T cells, phorbol esters are very effective at activating $p21^{ras}$. It is becoming clear that many of the effects of phorbol esters in T cells previously attributed to PKC are in fact $p21^{ras}$ mediated (50).

Much of the progress in understanding signal transduction pathways in T cells has been achieved because of coordinated efforts to link up immediate biochemical events triggered at the cell membrane with the transcription factors that control cytokine gene expression. Multiple transcription factors regulate the IL-2 gene including AP-1, NFκB, Oct-1, and nuclear factor of activated T cells (NFAT). IL-2 gene expression cannot be induced by a single signaling pathway; it requires the action of several pathways that integrate at the level of these transcription factors (51, 52). The function of $p21^{ras}$ in T cells has been explored using transient transfection protocols that examine the consequences of expressing mutated constitutively active or dominant inhibitory $p21^{ras}$ mutants on the activity of coexpressed reporter genes for different transcription factors. The $p21^{ras}$ oncogene p21-$^{\text{-v-Ha-ras}}$ is mutated at codon 12 (Ser > Val) and 59 (Ala > Thr). These mutations render the $p21^{ras}$ protein insensitive to negative regulation by Ras-GAPs such that it accumulates in cells in an "active" GTP bound state. p21-v-Ha ras, when expressed in T cells, can activate transcriptional factors such as AP-1 and also synergize with a calcium signaling pathway to activate nuclear factor of activated T cells (NFAT) and the IL-2 gene (53, 54). NFAT proteins are cytosolic in quiescent T cells, but they translocate to the nucleus in TCR-activated cells and combine with AP-1 to form a functional transcriptional factor complex (55). There are multiple isoforms of the NFAT cytosolic subunit: NFATc, NFATp, NFAT3, and NFAT4 (56, 57). These cytosolic NFAT proteins have different patterns of cellular expression but are thought to share one property in that their nuclear translocation is controlled by calcium-dependent signals. The different NFAT proteins also share the ability to interact with AP-1 to bind cooperatively to the composite NFAT/AP-1 site in the IL-2 gene (55). The contribution of $p21^{ras}$ in NFAT induction is thought

to reflect the involvement of Ras in the regulation of AP-1, although it is also possible that $p21^{ras}$ signals can induce posttranslational modifications of NFAT proteins and thus directly modify their transcriptional activity. Binding sites for NFAT are found in the enhancers of a number of cytokine genes including IL-2, IL-4, and TNFα (55). The ability of $p21^{ras}$ to regulate NFAT may thus be generally relevant to cytokine gene induction and not a specific pathway for the IL-2 gene.

The calcium signaling system that cooperates with $p21^{ras}$ for NFAT induction involves the calcium phosphatase calcineurin (58). The role of calcineurin in T cell activation was established in studies that probed the mechanism of action of the immunosuppressive drugs cyclosporin A and FK506 (52). These drugs when bound to their cell binding protein (immunophilins) inhibit calcium-mediated signaling pathways in lymphocytes. The FK506 or cyclosporin A/immunophilin complex can bind and sequester cytosolic calcineurin, which correlates with the inhibitory effects of these drugs on cytokine gene expression. The role of calcineurin in calcium-mediated signaling in T cell was supported further by experiments showing that overexpression of calcineurin could reverse the immunosuppressive effects of FK506 (58). Moreover, expression of a truncated constitutively active catalytic subunit of calcineurin could mimic the effect of calcium ionophores on T cell activation (59).

The regulatory effects of PMA on NFAT induction have suggested a regulatory role for PKC in NFAT function. There is additional evidence that PKC can contribute to NFAT induction because expression of constitutively active PKC ϵ or α mutants in T cells can synergise with calcium signals to stimulate NFAT transcriptional activity (60). It is established that TCR triggering activates PKC, and an obvious assumption for many years was that TCR induction of NFAT was PKC and calcium/calcineurin mediated (Figure 2). This assumption was supported further by observations that PKC activity is necessary for induction of the IL-2 gene (61). However, PKC inhibitors have no effect on TCR stimulation of NFAT or AP-1 (61). IL-2 gene induction requires the coordinate action of multiple transcription factors that include NFAT, AP-1, NFκB, and Oct-1; and PKC plays an essential role in NFκB induction in T cells, which could explain its role in IL-2 gene expression (Figure 2) (61). $p21^{ras}$ signaling pathways are clearly able to substitute for PKC signals for NFAT induction, and the TCR activates $p21^{ras}$ in a PKC-independent mechanism. The relative contribution of Ras signals for TCR induction of NFAT has been resolved by experiments with a dominant inhibitory $p21^{ras}$ mutant, N17ras. Expression of N17 ras inhibits the accumulation of active ras GTP complexes in T cells (62) and prevents TCR induction of NFAT (63), indicating that Ras signals are indispensable for NFAT function (Figure 2). It is clear however that $p21^{ras}$ is

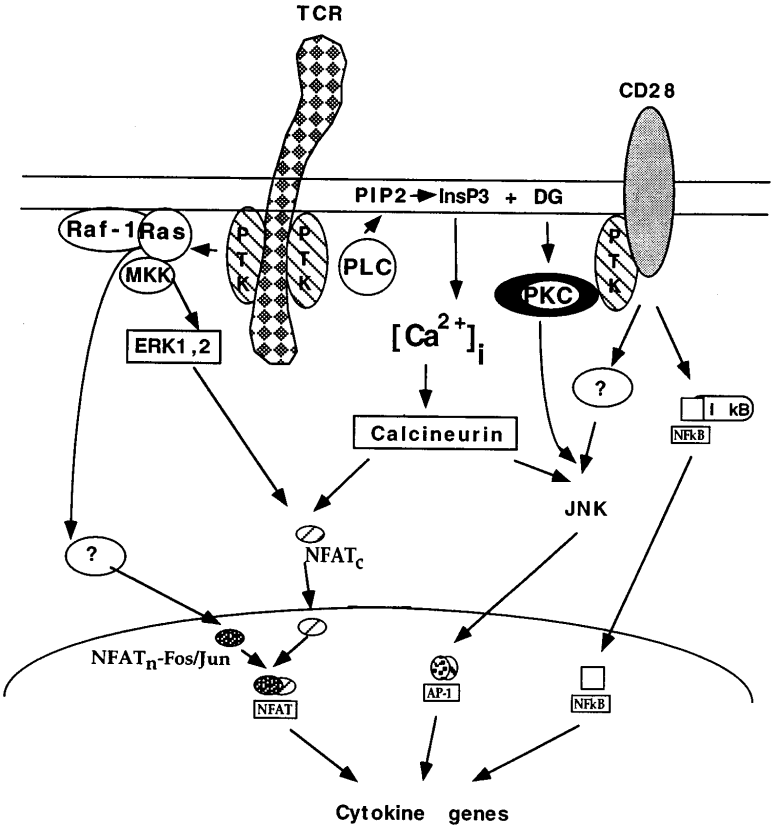

Figure 2

not required for all TCR functions, because expression of dominant negative ras in transgenic mice suppressed TCR-mediated positive selection and TCR-mediated proliferation of thymocytes in vitro but did not influence the negative selection of thymocytes (64).

THE REGULATION AND FUNCTION OF MAP KINASES IN T CELLS

The transmission of signals from $p21^{ras}$ to the nucleus is proposed to involve the regulation of the Map kinases (mitogen-activated protein kinases) ERK1 and ERK2 (65). Two intracellular pathways for ERK2 regulation co-exist in T cells: one mediated by Ras and the other by PKC (66) The TCR stimulates

both $p21^{ras}$ and PKC, but $p21^{ras}$ and not PKC couples the TCR to the regulation of MAP kinases (67). The MAP kinases are activated by a kinase cascade involving a MAP kinase kinase (MKK) that phosphorylates and stimulates the ERK1 and 2 kinases directly. The activity of the MKK is itself controlled by phosphorylation, and hence a MAP kinase kinase kinase (MKKK) plays a crucial role in the the regulation of ERK1 and 2 (4, 68). In many cells, Raf-1 has been identified as the MAPKKK that plays a key role in coupling $p21^{ras}$ and hence receptors that stimulate $p21^{ras}$ to the MAP kinases (69). The N-terminal regulatory domain of the serine/threonine kinase Raf-1 can interact directly with "activated" GTP bound $p21^{ras}$, and it has been suggested that the role of Ras is to recruit Raf-1 to the membrane where it is activated (68). In T cells, constitutively active Raf-1 can mimic the effect of activated Ras and synergise with calcium signals to induce the IL-2 gene (70, 71). A constitutively active Raf-1 can also mimic the TCR and stimulate ERK2; a dominant negative Raf-1 prevents TCR stimulation of ERK2 (72). These observations collectively suggest that Raf-1 is one effector molecule for $p21^{ras}$ in T cells.

One well-documented role for the MAP kinase ERK2 is in the phosphorylation and regulation of Elk1 (73, 74). Elk1 is one of a family of proteins that can form a ternary complex with the transcriptional-activator serum response factor and thus can play a key role in regulation of Fos gene expression. Accordingly, the role of ras in coupling the TCR to ERK2 and the ability of ERK2 to translocate to the nucleus where it can directly modulate transcriptional factors could explain the role of $p21^{ras}$ in TCR signal transduction. In particular, Fos proteins are components of the AP-1 complex that is clearly important for NFAT activity and cytokine gene induction. The link between Ras stimulation of ERK2 and Ras induction of lymphokine gene expression has not yet been fully explored, but there is preliminary data suggesting that not all Ras functions are mediated by the MAP kinase ERK1,2 cascade. For example, in transgenic mouse experiments, a dominant negative MKK can mimic inhibitory $p21^{ras}$ mutants and suppress positive selection of thymocytes (75). However, inhibitory Ras mutants prevent TCR-induced proliferation of thymocytes, whereas an inhibitory MKK mutant does not. Also, in transfection experiments in T cell lines, expression of a dominant negative MKK can abrogate NFAT induction analogous to the effects of dominant negative Ras, but expression of a constitutively active MKK cannot substitute for constitutively active Ras and induce AP-1 or synergise with calcium dependent signals to induce NFAT (Elisabeth Genot, Doreen Cantrell, unpublished data). These studies collectively suggest that the ERK1/2 pathway is not the sole Ras effector pathway in T cells. Accordingly, a major challenge for the future is to elucidate alternative Ras effector molecules in T cells.

REGULATION OF JUN KINASES BY TCR AND CD28

Two additional MAP kinase signaling pathways have been characterized that allow parallel regulation of the kinases JNK1,2 and MPK2/p38 (65). The role of the TCR in activation of MPK2/p38 is not clear, whereas the regulation of the Jun kinases JNK1,2 is now recognized to be a point of convergence for TCR and CD28 signaling pathways. Triggering of the costimulatory molecule CD28 activates cellular PTKs, and there are some cellular proteins that are common substrates for both TCR- and CD28-activated PTKs including $p95^{vav}$ (41). However, the activation of phospholipase C or the Ras/Raf-1/ERK2 signaling pathway is not a physiological response to CD28 triggering with its natural ligands B7-1 or B7-2. Rather CD28 regulates the activity of PtdIns 3–kinase and generates signals that synergise with TCR-derived signals to activate the MAP kinase JNK1 (76). The activation of JNK1 in T cells seems to require calcium/calcineurin signals, CD28 signals, and other TCR-derived signals. This is also true for cytokine gene expression, which suggests that Jun kinases are the crucial integration point for both TCR- and CD28-derived signals in T cell activation (Figure 2) (76). The Jun kinases are regulated by kinase cascade analogous to that described for the regulation of ERK1 and 2. Moreover, just as the GTPase $p21^{ras}$ is implicated in ERK1,2 activation in T cells, it is likely that the $p21^{ras}$-related GTPase Rac may be important in Jun kinase regulation in T cells (Elisabeth Genot, Doreen Cantrell, unpublished observations). The point of convergence of TCR and CD28 signals in the Jun kinase signaling cascade is not yet known, but it will be intriguing to establish whether Rac has a role in CD28 signal transduction that is analogous to the role of $p21^{ras}$ in TCR signaling. In this context, T cells express a variety of Ras- and Rac-related GTPases, including Rho and CDC42. These two low-molecular-weight G proteins are important for $p21^{ras}$-mediated transformation of fibroblasts, which in part reflects that they play an important role in the control of the actin cytoskeleton (77–79). Moreover, it is recognized that Rho may be equally as important as $p21^{ras}$ and Rac in the regulation of protein kinase cascades that culminate in transcription factor phosphorylation (80). Accordingly, it will be of interest to explore the signaling connections between $p21^{ras}$ and Rho and Rac in T cells in order to determine whether the $p21^{ras}$ control of cytokine genes involves Rho/Rac controlled signals.

PROTEIN COMPLEXES IN T CELLS

It is well documented that during T cell activation there are multiple protein complexes formed by interactions between TCR-induced tyrosine phosphoproteins and the SH2 domains of various effector tyrosine kinases and/or adapter

proteins (Figure 3). For example, the TCR-coupled tyrosine kinase ZAP70 binds to tyrosine phosphorylated ITAMs in the TCR complex via its tandem SH2 domains. In TCR-activated cells ZAP70 is tyrosine phosphorylated and can itself interact with the the SH2 domains of the molecules such as Vav or the PTK p56lck (81, 82). Thus the importance of ZAP70 in T cell activation may not only reflect ZAP70 functions as a tyrosine kinase; rather, the 'adapter' role for ZAP70 may be equally important. Another molecule with the potential for pleotropic functions in T cells is the TCR-induced p36 tyrosine phosphoprotein that appears to be an adapter that links TCR-regulated PTKs to both phospholipase C-γ-1 and Grb2 (83). In this context, Grb2 function in T cells should not be considered solely in terms of Sos/p21^{ras} regulation. Thus, Grb-2 SH3 domains can bind molecules other than Sos and may therefore regulate multiple effector pathways. In particular, recent studies have identified at least three other potential Grb2 SH3 binding effector proteins in T cells: a 75-kDa and 116-kDa protein, PtdIns 3–kinase and the proto-oncogene c-cbl (16, 84–86). The function of these proteins is not known, but they are all substrates for TCR-activated PTKs. The 75-kDa Grb2 SH3 binding protein in T cells has recently been characterized and is itself apparently an adapter molecule (87). Thus, p75 has proline-rich peptide sequences that could explain its interaction with Grb2 SH3 domains. p75 also has an SH2 domain that can bind tyrosine phosphoproteins in TCR-activated cells. Finally, when p75 is tyrosine phosphorylated in

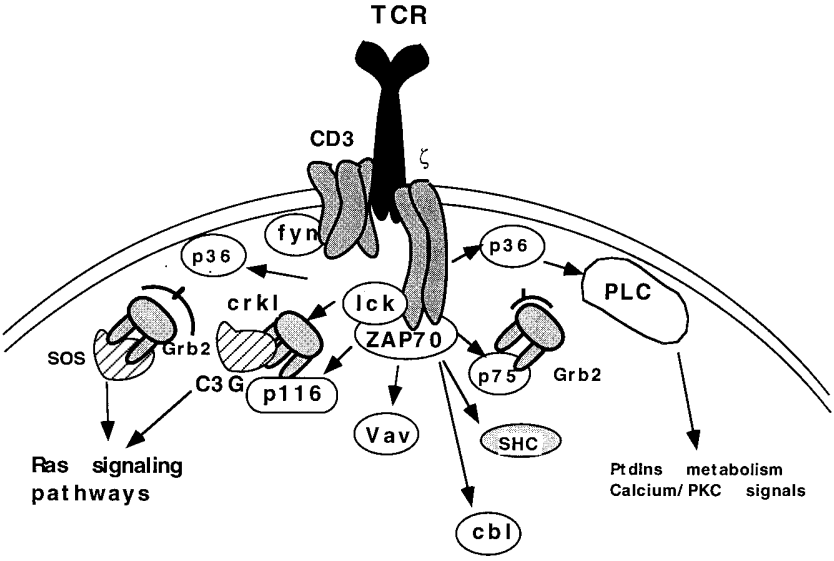

Figure 3

TCR-activated cells, it could bind to the SH2 domains of other molecules. p75 could thus form a scaffold for the formation of protein complexes in T cells.

The adapter Grb2 thus appears to be an important component of TCR signal transduction mechanisms but is not similarily involved in the CD28 costimulatory pathways. The 36-kDa Grb2 SH2 binding protein and the 75-kDa Grb2 SH3 binding protein seem to be selective substrates for the TCR-activated PTKs rather than the CD28 PTKs. Conversely, CD28 can induce tyrosine phosphorylation of a 62-kDa adapter molecule, and this appears to be a unique response to CD28 ligation that is not shared with the TCR (Jacques Nunes, Doreen Cantrell, unpublished data). p62, like Grb2, is now recognized as a multifunctional adapter protein and can probably link receptor coupled PTKs to several downstream signal transduction pathways (88). The differential regulation of adapter proteins by the TCR and CD28 may thus partially explain the divergence of TCR and CD28 signal transduction mechanisms.

FUTURE DIRECTIONS OF TCR SIGNAL TRANSDUCTION RESEARCH

Recently, partial tyrosine phosphorylation of the TCR complex and recruitment but not activation of ZAP70 has been described during the induction of T cell anergy (89, 90). One source of variability in TCR signaling could originate from the ITAMS, which all have the duplicated YXXL sequence but vary quite markedly in their intervening amino acid sequences; they thus have the potential to interact with different cytosolic effectors. In this context, chimeric receptors in which the receptor cytoplasmic tail comprises either the ζ or ϵ ITAM are able to induce calcium mobilization and tyrosine phosphorylation pathways (2, 91, 92), but they induce different patterns of cellular tyrosine phosphorylation, indicating that the different ITAMS may have unique functions (91). One of the major challenges for future studies will be to understand how the TCR is able to channel its signaling responses. There will undoubtedly be great emphasis on the mechanisms that the TCR might use to generate signaling diversity.

Studies of TCR signaling to date have revealed the enormous signal transduction potential of the TCR. In the last year, the sophistication of current technology has facilitated the identification of numerous potentially important TCR-triggered biochemical events. There is very little understanding of the functions of many of the protein complexes that are targets for TCR signals. However, the technology used to explore $p21^{ras}$ and tyrosine kinase functions will undoubtedly be used in the future to rectify this deficiency. It will be important in the future to extend the characterization of the role of the different TCR signals beyond the analysis of cytokine gene induction, because this is only one function of the TCR in T cell biology. The TCR is also important in

the following responses: the postive and negative selection of thymocytes, the regulation of T cell cytotoxicity via the control of the secretion of cytotoxic mediators such as perforin; the regulation of T cell adhesion via regulation of integrins and their binding partners. Questions concerning the signal transduction mechanisms of these different TCR functions are equally important as those concerning the regulation of cytokine genes.

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ANTIGEN SAMPLING ACROSS EPITHELIAL BARRIERS AND INDUCTION OF MUCOSAL IMMUNE RESPONSES

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ABSTRACT

Epithelial barriers on mucosal surfaces at different sites in the body differ dramatically in their cellular organization, and antigen sampling strategies at diverse mucosal sites are adapted accordingly. In stratified and pseudostratified epithelia, dendritic cells migrate to the outer limit of the epithelium, where they sample antigens for subsequent presentation in local or distant organized lymphoid tissues. In simple epithelia, specialized epithelial M cells (a phenotype that occurs only in the epithelium over organized lymphoid follicles) deliver samples of foreign material by transepithelial transport from the lumen to organized lymphoid tissues within the mucosa. Certain pathogens exploit the M cell transport process to cross the epithelial barrier and invade the mucosa. Here we review the features of M cells that determine antigen and pathogen adherence and transport into mucosal lymphoid tissues.

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ORGANIZATION OF THE MUCOSAL IMMUNE SYSTEM

The mucosal surfaces of the gastrointestinal, respiratory, and urogenital tracts have a combined surface area of at least 400 m^2, covered largely by a single layer of epithelial cells. These cellular barriers face environments rich in pathogens, some of which have developed effective mechanisms for colonization of epithelial surfaces and invasion of mucosal tissues. In defense, mucosal tissues are heavily populated with cells of the immune system: It is estimated that the intestinal lining contains more lymphoid cells and produces more antibodies than any other organ in the body (1, 2). Antibodies produced in mucosal tissues are difficult to quantitate, however, since they consist primarily of polymeric IgA that is continuously exported into secretions.

The mucosal immune system consists of specialized local inductive sites (the Organized Mucosa-Associated Lymphoid Tissue or O-MALT) and widespread effector sites (the Diffuse Mucosa-Associated Lymphoid Tissue or D-MALT), both of which are separated from mucosal surface antigens by epithelial barriers (3). Some mucosal tissues such as the vagina have no local O-MALT but rely on antigen uptake and transport into lymph nodes that drain the mucosa (4). In either case, the first step in the induction of a mucosal immune response is the transport of antigens across the epithelial barrier. Following antigen processing and presentation in inductive sites, IgA-committed, antigen-specific B lymphoblasts proliferate locally and then migrate via the bloodstream to local and distant mucosal and secretory tissues. There they differentiate primarily into polymeric IgA-producing plasma cells, important components of D-MALT (5, 6; reviewed in 3). Dimeric or polymeric IgA antibodies are transported across epithelial cells into glandular and mucosal secretions via receptor-mediated transcytosis (reviewed in 7, 8).

O-MALT, characterized by mucosal lymphoid follicles, occurs in the tonsils, bronchi, and intestines (9, 10). Single follicles occur along the entire length of the gastrointestinal (GI) tract, with increasing frequency in the colon and rectum (11, 12). Aggregated follicles occur in the lingual and palatine tonsils and adenoids of the oral- and nasopharynx, the Peyer's patches in small intestine, and the appendix (9–12). A typical intestinal lymphoid follicle has a B lymphocyte–rich peripheral zone and a germinal center, and it is separated from the epithelium by a dome-shaped "corona" containing B cells, $CD4^+$ T cells, and antigen-presenting dendritic cells and macrophages (13). Where follicles are aggregated, they are separated from each other by interfollicular zones rich in T cells and containing high endothelial venules where extravasation of circulating lymphocytes occurs (14). There is abundant evidence that O-MALT plays a major role in antigen sampling and generation of lymphocytes, including specific IgA effector B cells, memory B cells, and T cells. This involves

active lymphocyte proliferation, local production of certain cytokines, and continuous cellular trafficking (15–17). In certain developing animals, O-MALT may also play a role in shaping the repertoire of immunoglobulins and T cell receptors (18).

MECHANISMS OF ANTIGEN TRANSPORT ACROSS EPITHELIAL BARRIERS

Epithelial linings of different mucosal surfaces differ dramatically. Multilayered squamous epithelia line the oral cavity, pharynx, esophagus, urethra, and vagina. The intestinal mucosa is covered by only a single cell layer, and the airway lining varies from pseudostratified to simple epithelium. These diverse epithelia are not impenetrable barriers, but rather are cell assemblies that control cross-talk between the exterior and the interior using multiple antigen sampling strategies (Figure 1). In stratified and pseudostratified epithelia, antigen-processing dendritic cells serve as motile "scouts" that move into the epithelium, obtain samples of luminal antigens, and migrate back to local or distant organized lymphoid tissues. In simple intestinal and airway epithelia whose intercellular spaces are sealed by tight junctions, specialized epithelial M cells deliver samples of foreign material by transepithelial transport from the lumen to organized lymphoid tissues within the mucosa.

Sampling of pathogens by either mechanism—dendritic cell or M cell—has inherent risks in that pathogens can exploit such transport mechanisms to cross the epithelial barrier and invade the body. In the case of dendritic cells in stratified epithelia, there is little information available about their role in mediating infection, but one may assume that the lumenal contacts of these motile cells present a very limited and transient opportunity to pathogens. In contrast the broad, nonmotile M cells of the intestine are highly exposed to the lumen of the gut and are relatively accessible to attachment and invasion of pathogens. The risk of invasion and mucosal or systemic disease appears to be reduced by the close and complex interactions of the follicle-associated epithelium with professional antigen processing and presenting cells, and by the organization of mucosal lymphoid tissues immediately under the epithelium.

Antigen Sampling Across Stratified Epithelia

BARRIER FUNCTION OF STRATIFIED EPITHELIA Stratified, nonkeratinized or parakeratinized epithelia lack tight junctions but provide a permeability barrier by secretion of a "membrane-coating" glycolipoprotein substance into the narrow intercellular spaces of the lower stratified layers (19). The close association of epithelial cells along with secreted intercellular material renders the oral epithelium impermeable to protein tracers and large cations, and it effectively

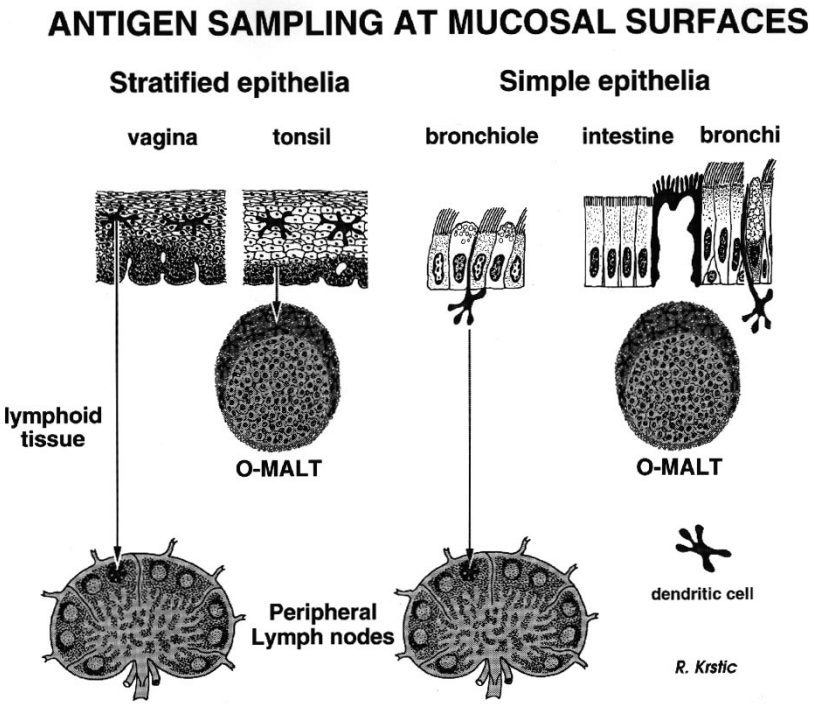

Figure 1 Antigen sampling across the stratified epithelia of the oral cavity and vagina and the epithelia of the airways is accomplished by migratory dendritic cells that carry antigens to local organized mucosa-associated lymphoid tissues (O-MALT) or to distant lymph nodes. Antigen transport across simple intestinal and bronchial epithelia is accomplished by M cells that deliver samples of foreign material to local O-MALT.

excludes most infectious agents and vaccines. Regional variations in the thickness, surface cell phenotypes, and protein expression in stratified squamous epithelia are determined by interplay of genetic factors with the local environment. In the female genital tract, hormonal fluctuations over the course of the menstrual cycle have dramatic effects on the stratified epithelium of the vagina and exocervix, affecting epithelial thickness, endocytic activity of epithelial cells, and the distribution of dendritic cells (20). Although proteins can be taken up by stratified epithelial cells (21), such epithelia have no mechanism for directional transcytosis. Nor can proteins, other macromolecules, or microbes passively diffuse through the stratified epithelia of the female reproductive tract or the oral cavity. Thus, samples of intact foreign antigens from these mucosal surfaces are obtained through the activities of motile dendritic cells (Langerhans cells)

and macrophages. The fact that sampling of antigens across stratified epithelia requires close collaboration between epithelial and dendritic cells explains why the effectiveness of vaginal vaccines varies widely, depending on the stage of the menstrual cycle as well as the vaccine or vector used (4, 22).

INTRAEPITHELIAL DENDRITIC CELLS AND MACROPHAGES

Intraepithelial dendritic cells, equivalent in function to the Langerhans cells originally described in skin, are found in the oral cavity (particularly in the tonsils; 23, 24), in the stratified epithelium of the vagina (25, 26), and within the pseudostratified airway epithelium (27). Dendritic cells of mucosal surfaces can serve as antigen-presenting cells after migration out of mucosal tissues to draining lymph nodes (28), or in the case of tonsils, they can present antigens locally to cells of mucosal lymphoid follicles (24) (Figure 1). Whether the immunological outcome of antigen sampling by dendritic cells and macrophages in organized tonsillar lymphoid tissue differs from the outcome of dendritic cell sampling in the general buccal epithelium is not known.

In the airway epithelium, dendritic cells form a contiguous network with up to 700 dendritic cells per mm^2 (27). Under normal conditions these cells represent the major MHC class II–bearing, antigen-presenting cells of the airways, suggesting that they constitute a first line of defense against inhaled antigens. Renewal of the airway dendritic cell population from bone marrow is balanced by migration of cells to draining lymph nodes with a half-life of only 2 days, similar to that reported for the dendritic cell population of the gut wall (28). During acute inflammatory responses, dendritic cell precursors are recruited to the respiratory epithelium where they differentiate and mature into resident dendritic cells (29).

In sampling the mucosal surface environment, dendritic cells run the risk of infection. Their antigen uptake and migratory activities, coupled with their ability to form close associations with T cells (30), may lead to replication and dissemination of T cell–tropic viruses that contact mucosal surfaces. Dendritic cells isolated from skin were infected after exposure to HIV in vitro, and interaction of dendritic cells and memory T cells resulted in efficient infection and viral production by the T cells (31, 32). Dendritic cells in intact vaginal tissue of monkeys were found to be infected after mucosal exposure to SIV (33). Since other transepithelial transport mechanisms such as M cells seem to be absent in the female reproductive tract (4), intraepithelial dendritic cells of the vagina and ectocervix are currently thought to be important in transmission of HIV as well as in the mucosal immune response to vaginal vaccines.

Antigen Sampling Across Simple Epithelia

BARRIER FUNCTION OF SIMPLE EPITHELIA Most of the vast mucosal surfaces of the gastrointestinal tract and airways are lined by a single layer of epithelial cells sealed by tight junctions that exclude peptides and macromolecules with antigenic potential (34). Damaged or aged epithelial cells are replaced from a pool of precursor cells that are sequestered in crypts in the intestine (35) or maintained as basal cells throughout the epithelium in the airways. Uptake of macromolecules, particulate antigens, and microorganisms across healthy simple epithelia can occur only by active vesicular transport across epithelial cells, and this is restricted by multiple mechanisms (36). Many antigens and microorganisms are prevented from contact with epithelial surfaces by local secretions containing mucins and secretory IgA antibodies (3, 37). In the intestine, the epithelial cells themselves are well equipped to face a pathogen-rich foreign environment. The apical surfaces of enterocytes are modified by rigid, closely packed microvilli—the brush border—coated with a thick layer of large membrane-anchored, negatively charged mucin-like molecules called the glycocalyx (38, 39). The glycocalyx constitutes both a diffusion barrier and a highly degradative microenvironment. This is owing to entrapped pancreatic enzymes and membrane-bound hydrolytic enzymes (40) that impede the access of macromolecular aggregates, particles, and microorganisms to the apical cell membrane. Intestinal enterocytes nevertheless are able to take up antigens by endocytosis (36). They express MHC class I and/or class II molecules constitutively or in response to cytokines (41, 42). Enterocytes are able to present antigen in vitro, but the role of these cells in antigen presentation in vivo remains controversial (43, 44).

LOCAL EPITHELIAL SPECIALIZATIONS The lymphoid follicles of O-MALT are separated from the lumen by a specialized follicle-associated epithelium (FAE) containing enterocyte-like cells and antigen-transporting M cells, a highly specialized phenotype located exclusively at these sites (Figure 2). M cells transport macromolecules, particles, and microorganisms directly into the special microenvironment of mucosal lymphoid follicles, and this seems to be an important first step in initiation of a secretory immune response (45).

THE FOLLICLE-ASSOCIATED EPITHELIUM

Unique Features of the FAE

A striking feature of the FAE in both the GI tract and airways is the total absence of receptors for polymeric immunoglobulin, indicating an inability to secrete IgA (46, 47). This is consistent with the observation that O-MALT

contains precursors of IgA B cells but is not a site of terminal differentiation or IgA production (5, 6). A local lack of surface-associated secretory IgA may facilitate access of antigens to the mucosal surface. Paradoxically, M cells in experimental animals have been shown to bind and transport IgA and IgA immune complexes from the lumen into the intraepithelial pocket (48). We have suggested that uptake of immune complexes by M cells could boost or modulate mucosal immune responses, but the fate of such complexes or the significance of M cell interactions with luminal antibodies is unknown.

Interactions of M Cells with Lymphocytes and APC

M cells of the FAE are specialized for transepithelial transport across the epithelial barrier. The M cell basolateral surface is deeply invaginated to form a large intraepithelial "pocket" into which transcytosed particles and macromolecules are delivered (45). This unique structural modification is supported by an extensive array of intermediate filaments and is lined by a distinct "pocket domain"

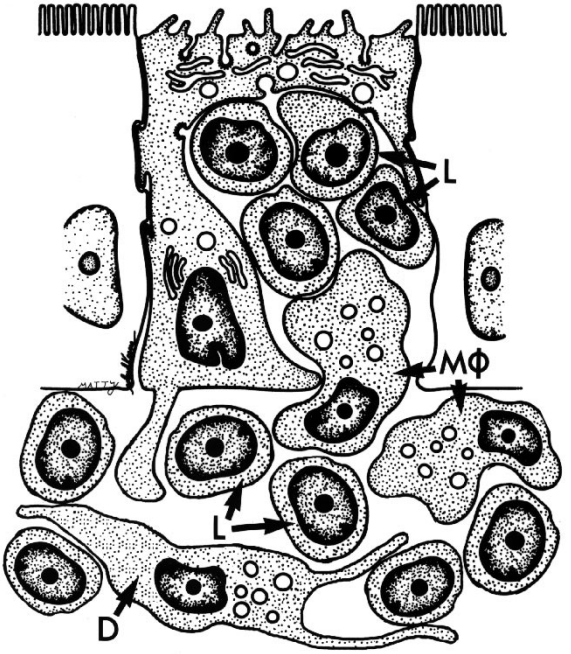

Figure 2 Diagram of an M cell in the intestinal follicle-associated epithelium. The intraepithelial pocket contains B and T lymphocytes (L) and an occasional macrophage (Mϕ). Below the epithelium are macrophages and dendritic cells (D) and many T and B lymphocytes.

of the plasma membrane (49). The pocket and its content of immigrant cells is a cardinal feature of the M cell. Specific subpopulations of lymphocytes migrate into the pocket and associate closely with the pocket membrane, forming apparent adhesion sites. The recognition and adherence mechanisms that direct and retain lymphocytes and occasional macrophages in the M cell pocket are unknown, but it seems safe to assume that specific epithelial and lymphocyte adhesion molecules, analogous to but distinct from the IEL-enterocyte adhesion partners (50), mediate these events. M cells also have basal processes that extend 10 μm or more into the underlying lymphoid tissue where they could make direct contact with lymphoid or antigen-presenting cells (51). It is tempting to think that such contacts play a role in the induction of the unique M cell phenotype or in the processing and presentation of antigens after M cell transport.

The immigrant cells in M cell pockets have been identified by immunocytochemistry of intestinal Peyer's patches of rodents, rabbits, and humans (52–55). In all species, both B and T lymphocytes were present along with a small number of macrophages. Most of the T cells were $CD4^+$, and none displayed the gamma/delta T cell receptor typical of villus IELs, although in rabbit there were cells lacking both CD4 and CD8 (54). Human M cell–associated T cells displayed the marker antigen CD45RO typical of memory cells, although in some specimens naive T cells were observed (55). Most T cells expressed the early activation marker CD69, and most (but not all) were $CD4^+$. The fact that B cells in the pocket express the "naive" cell marker CD45RA along with HLA-DR suggests that the M cell pocket is a site of interaction of T cells with antigen-presenting B cells. The B cells contained IgM but neither IgG nor IgA, suggesting that B memory cells and initial B cell differentiation also may occur here. Since the B cells in M cell pockets are of the same types as the subepithelial B cells associated with the underlying follicle, it has been suggested that B lymphoblast traffic into the M cell pocket may allow continued antigen exposure and extension and diversification of the immune response (55). With the abundance of B lymphoblasts, helper T cells, and APC immediately below the FAE, what is the purpose of antigen presentation in the M cell pocket? One possibility is that the cells in the pocket can interact early with incoming antigens, in an environment sequestered from the modulating influence of systemic humoral immunity.

Below the epithelium of the dome lies an extensive network of macrophages and dendritic cells intermingled with $CD4^+$ T cells and B cells from the underlying follicle (56: Figure 2). These cell populations are presumably active in uptake and killing of incoming pathogens as well as in processing, presentation, and perhaps storage of antigens. There is little information available concerning

the fates of specific antigens, pathogens, and vaccines in the FAE, subepithelial tissue, or follicle. Viruses and bacteria that use the M cell as an invasion route can infect and destroy M cells and can infect nearby macrophages and lymphocytes, as discussed below. On the other hand, killed microorganisms and tracer antigens have been seen in macrophages in the M cell pocket and the subepithelial tissue, and it is likely that such antigens are processed and presented both within the mucosa and in draining lymph nodes.

UPTAKE OF ANTIGENS BY M CELLS

M Cell Luminal Surfaces

APICAL MEMBRANE ORGANIZATION The M cell apical surface differs from that of intestinal absorptive cells in the lack of a well-organized brush border and the presence of large endocytic domains that function in uptake of macromolecules, particles, and microbes (57, 58). The organization of the apical cytoskeleton in M cells is distinct: The cells generally lack long microvilli with uniform microvillar cores, and the actin-associated protein villin that is confined to such cores in enterocytes is diffusely distributed in M cells (S Kerneis, A Bogdanova, E Colucci-Guyon, J-P Kraehenbuhl, E Pringault, submitted) reflecting their modified microvillar organization and perhaps their ability to respond to adherence of microorganisms with ruffling and phagocytosis.

There is little information about the composition of apical membranes of M cells, but they do have distinct features that can be used as identification markers. M cells lack the uniform thick glycocalyx seen on enterocytes, although their apical membranes display abundant glycoconjugates in a cell coat that varies widely in thickness and density (59). M cell membranes contain the glycolipid GM1 and bind cholera toxin, either in soluble form or on small (10–12 nm gold) particles. However, the M cell glycocalyx is sufficient to prevent access of cholera toxin-coated 1-micron particles to the GM1 receptor (A Frey, W Lencer, K Giannasca, R Weltzin, MR Neutra, unpublished data). Alkaline phosphatase, abundant on enterocytes, is often (but not always) reduced or absent on M cells (60, 61). In several species, their surface membrane displays distinct oligosaccharides that may be important for selective M cell adherence of certain enteric pathogens. However, neither the microbial surface molecules that mediate adherence of bacteria or viruses, nor the M cell surface molecules that serve as receptors have been identified.

MEMBRANE GLYCOCONJUGATES Lectins and antibodies have revealed that M cell glycosylation patterns can be used to distinguish them from enterocytes. For example, two lectins (UEA I and WBA II), which recognize a range of carbohydrate structures containing α(1–2)-fucose, selectively stain M cells

in the Peyer's patches of BALB/c mice (51, 62, 63). These lectins label M cell apical membranes, and also intracellular vesicles and basolateral membranes, including the pocket domain (51). Other lectin and antibody probes revealed variations in the glycosylation patterns of individual M cells within a single FAE (51). This diversity might expand the possible microbial lectin-M cell interactions of the local FAE and allow the M cells to "sample" a wide variety of microorganisms. Furthermore, glycoconjugates expressed on M cells in other intestinal regions (cecum, appendix, colon, and rectum), in other mouse strains, and in other species including monkey and human (F Zhou, P Giannasca, K Giannasca, A Leichtner, M Neutra, unpublished data) are distinct from those in BALB/c mouse Peyer's patches (51, 63–65).

Regional differences in M cell surfaces must be taken into account when designing mucosal vaccines or using region-specific live vectors. There is mounting evidence that the location in which M cell transport and inductive events occur has a profound influence on the subsequent regional distribution of specific IgA plasma cells and IgA secretion (66). This was originally demonstrated in studies using poliovirus: A secretory immune response against poliovirus was demonstrated in the colon but not in the nasopharynx following immunization of the distal colon (67). Other studies showed that secretory immune responses to Sendai virus were concentrated in either the digestive tract or the airways, but not in both, when administration of antigen was carefully restricted either to the stomach or the trachea (68). Similarly, cholera toxin (CT) instilled into proximal intestine, distal intestine, or colon evoked the highest levels of specific antitoxin secretory IgA in the segment of antigen exposure (69). To analyze local IgA responses more precisely, we developed "wicks" made of an absorbent filter material for direct retrieval of secretions associated with mucosal surfaces of the gastrointestinal system and vagina of mice (70). After immunizing mice via different mucosal routes with cholera toxin (70) or with a recombinant Salmonella vector expressing a foreign antigen (71), only the rectal immunization route produced high levels of specific sIgA in the mucus coating the rectum and distal colon. Lymphoid follicles with M cells are particularly numerous in the distal colonic and rectal mucosa of humans (11, 12). The surface characteristics of these M cells are relevant to future design of mucosal vaccines against HIV.

Endocytosis and Transepithelial Transport

Adherent macromolecules or particles bound to the apical plasma membranes of M cells are efficiently endocytosed or phagocytosed (72, 73) and can be released at the pocket membrane within 10–15 min. M cells use multiple endocytic mechanisms for uptake of macromolecules, particles, and microorganisms. Adherent viruses and macromolecules are taken up by adsorptive

endocytosis via clathrin-coated pits and vesicles (72). Nonadherent materials are taken up by fluid-phase endocytosis in either coated or uncoated vesicles (74, 75). Large adherent particles and bacteria trigger phagocytosis, involving the extension of cellular processes and the reorganization of the submembrane actin network (76). Each of these uptake mechanisms results in transport of foreign material into endosomal tubules and vesicles and large multivesicular bodies in the M cell apical cytoplasm and to their subsequent release by exocytosis into the pocket. The large vesicles contain the late endosome/lysosome membrane marker lgp120, generate an acidic internal milieu (77), and also contain the endosomal protease cathepsin E (78). Although MHC class II antigens on M cell membranes have been documented in subpopulations of M cells of some species (77), it is not known whether M cells participate in the processing and presentation of antigens.

ONTOGENY OF THE FAE AND M CELLS

Epithelial Renewal in the Intestine

The cells of the FAE, like all intestinal epithelial cells, are derived from stem cells in the crypts. In adult small intestine, each crypt is a clonal unit harboring a ring of anchored stem cells near the crypt base that give rise to multiple cell types that migrate upward in columns onto several adjacent villi (35, 79, 80). The epithelium of each villus is thus derived from several surrounding crypts. Similarly, renewal of the FAE is dependent on the proliferation of cells located in the crypts surrounding the mucosal lymphoid follicles (Figure 3). The follicle-associated crypts are unusual in that they contain two distinct axes of migration/differentiation originating from the same ring of crypt stem cells: Cells on one wall of the crypt differentiate into absorptive enterocytes, goblet cells, and enteroendocrine cells that migrate onto villi, and these cells acquire secretory functions and express receptors for polymeric immunoglobulin while still within the crypt (8). In contrast, cells on the opposite wall of the same crypt fail to express these receptors and move onto the dome where they acquire the features of M cells and distinct follicle-associated enterocytes (46, 59). While still in the crypt, some of these cells show M cell–like lectin-binding patterns (51). As they emerge from the crypt, differentiating M cells begin endocytic activity, fail to assemble brush borders, and acquire intraepithelial lymphocytes and the characteristic pocket.

Induction of M Cells

There is indirect evidence that cell contacts and/or soluble factors from mucosal lymphoid follicles play an important role in induction of FAE and M cells. The fact that the follicle-facing side of follicle-associated crypts shows

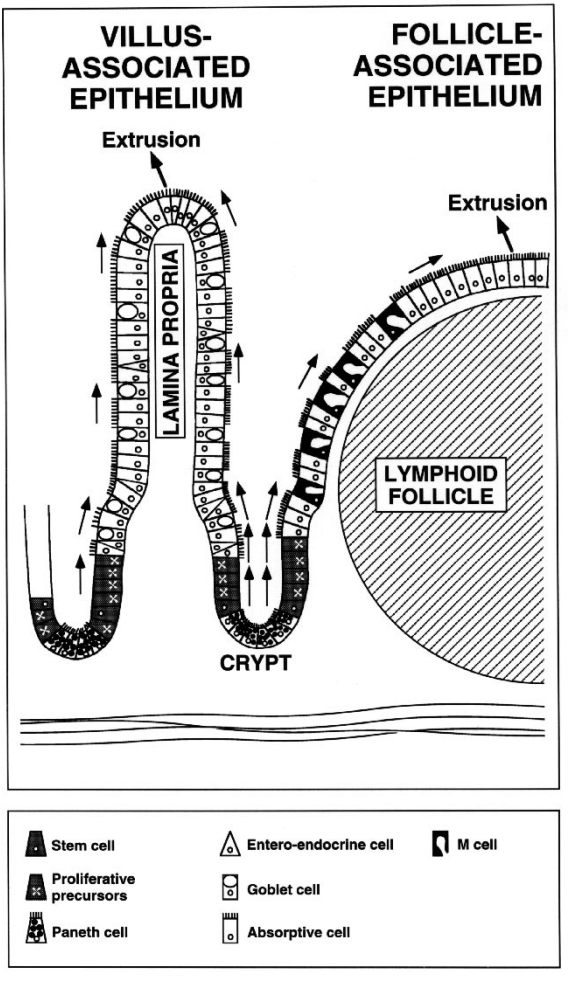

Figure 3 Renewal and differentiation of the follicle-associated epithelium in the small intestine. Crypts adjacent to lymphoid follicles contain stem cells that differentiate on one side into absorptive enterocytes, goblet cells, and enteroendocrine cells that migrate onto villi. On the other side of the same crypt, cells differentiate into M cells and distinct follicle-associated enterocytes that migrate into the dome over the lymphoid follicle.

distinct features including lack of Paneth cells, M cell–like glycosylation patterns, and lack of polymeric immunoglobulin receptors suggests that factors from O-MALT may act very early in the differentiation pathway, inducing crypt cells to commit to FAE phenotypes. On the other hand, factors or cells from the follicle or the lumen may also act later, to convert some of the FAE enterocyte-like cells to antigen-transporting M cells. The possibility of enterocyte–M cell conversions is supported by the observation that cells with both enterocyte and M cell features are present in FAE (59, 78) and that M cell numbers have been seen to increase within hours after Salmonella challenge (C Nicoletti, personal communication). Intestinal epithelial cells release cytokines in response to bacterial invasion (81); one could therefore imagine that bacterial colonization or invasion of FAE enterocytes results in epithelial cell signal transduction events which influence underlying lymphoid cells, and that the latter cells induce conversion of local enterocytes to the M cell phenotype. It is possible that this induction involves direct lymphocyte-epithelial cell contacts, including lymphocyte migration into the epithelium.

The importance of lymphoid cells in induction of the FAE is supported by the fact that injection of heterologous Peyer's patch cells into the submucosa of normal mice resulted in local assembly of a new lymphoid follicle and de novo appearance of FAE (82). Immunodeficient SCID mice lack mucosal follicles and lack identifiable M cells, but these mice develop follicles with overlying FAE and M cells after reconstitution of O-MALT with Peyer's patch cells from normal mice (83). Heterologous cell preparations enriched in B lymphocytes were most effective in this regard. In contrast, T cell–deficient nude mice have small Peyer's patches with FAE and M cells (84). Induction of O-MALT also follows inflammation: An inflamed ileal mucosa may contain increased O-MALT and an increased number of M cells in the FAE (85). The microflora of the intestinal lumen may influence differentiation of FAE and M cells by inducing assembly of O-MALT. Germ-free mice have a reduced number of Peyer's patches even though they are fully immunocompetent, but lymphoid follicles and M cells increase in number after transfer to a normal animal house environment (86). Repopulating germ-free mice either with a single nonpathogenic microorganism (*Clostridium indolis*) (S Kerneis, E Pringault, unpublished data) or with a single pathogen (*Salmonella typhimurium*;–87) was sufficient to restore the normal number of Peyer's patches with FAE-containing M cells. The three-way interaction of epithelium, microorganisms, and lymphoid cells seen in the FAE provides a dramatic demonstration of the phenotypic plasticity of the intestinal epithelium.

EXPLOITATION OF M CELLS BY PATHOGENS

Binding and uptake of enteric microorganisms by M cells appear to allow efficient sampling in inductive sites of the mucosal immune system. Under ideal circumstances, transport by M cells and the resulting secretion of antimicrobial sIgA antibodies limit the intensity or duration of mucosal disease and prevent reinfection (88). However, this mechanism of recognition and transport also increases the risk of infection and disease. Pathogenic microorganisms have developed diverse strategies for invasion via mucosal surfaces, including selective adherence to M cells. Bacteria and viruses that exploit M cell transport activity can rapidly infect the mucosa and/or spread systemically (76). Because distinct sets of biochemical tactics are involved, the interactions of pathogens with M cells vary widely, from simple transit to destruction of the FAE. Viruses are relatively simple molecular packages that rely on adherence to exploit the normal endocytic and transport activities of M cells. Bacteria, in contrast, use more complex strategies; for example, they can enzymatically alter the host cell surface, initiate signal transduction events that promote internalization, and recruit inflammatory cells, which in turn alter the epithelial barrier.

Viruses

REOVIRUS Exploitation of M cell transport by viruses was first recognized in studies of the mouse pathogen reovirus (89). Reovirus is transmitted orally and is processed by digestive proteases in the intestinal lumen. Proteolytic digestion does not inactivate the virus; rather, it remodels the viral outer capsid and activates the virus by releasing the major protein $\sigma 3$, cleaving the $\mu 1$c protein, and inducing extension of the viral hemagglutinin $\sigma 1$ (90, 91). Studies in our laboratory showed that intraluminal proteolytic processing is also required for M cell adherence (92). Although the M cell adhesin used by reovirus has not been identified, after luminal passage only the protease-resistant outer capsid protein $\mu 1$c and the extended $\sigma 1$ protein are available to bind to M cells. Reovirus recognizes mouse M cells not only in the Peyer's patches but also in the colon (93) and the airways (94), and it binds to rabbit as well as mouse M cells. In mice, reovirus uses the M cell to gain access to its target cells, particularly macrophages and neurons, in the mucosa. However, the virus can also invade intestinal epithelial cells from the basolateral side and can infect M cells during or after transport, causing selective loss of M cells from the FAE (95).

POLIOVIRUS Poliovirus type 1 and the attenuated Sabin strain selectively adhere to human M cells (96). The molecular structure and pathogenesis of poliovirus in humans shows some intriguing parallels with reovirus in mice, in that it enters by the oral route and proliferates in Peyer's patches before

spreading systemically (97). The fact that poliovirus exploits M cell transport to cross the epithelial barrier greatly enhances its potential importance as a candidate oral vaccine vector for delivery of foreign antigens in humans, either as live recombinant viral particles (98) or as inert virus-like particles (99). The receptor used by poliovirus to infect neuronal target cells has been identified, cloned, and used to generate transgenic mice that express the receptor and that become infected after injection of virus (100). They are not infected when challenged orally, however, indicating that the virus uses a different, unknown receptor to bind to M cells (V Racaniello, personal communication).

MOUSE MAMMARY TUMOR VIRUS Mouse mammary tumor virus (MMTV), a retrovirus that is transmitted to newborn mice via mother's milk, has to cross the intestinal epithelial barrier to infect lymphocytes, a key step for viral spread to the mammary gland (101). Although intestinal absorptive cells in suckling mice are highly endocytic (57) and MMTV proteins are found in all epithelial cells of the gut in pups fed infected milk (102), the earliest indicator of infection is retroviral DNA that is detectable only in the mucosa of Peyer's patches (103). This indicates that the virus first crosses the follicle-associated epithelium and infects Peyer's patch lymphocytes. As early as 5 days after birth, the infection leads to a superantigen response in the Peyer's patches, but not in other lymphoid organs draining the intestine. Later, viral DNA can be detected in mesenteric lymph nodes and finally in all lymphoid organs (103). Recently we have shown that MMTV can infect adult mice via the nasal or the rectal route (D Velin, H Acha-Orbea, J-P Kraehenbuhl, unpublished data). Although M cells have not been directly demonstrated to be the portal of entry of MMTV, the fact that M cells are present in Peyer's patch, nasal, and rectal inductive sites makes it likely that this retrovirus exploits the M cell to cross various epithelial barriers.

HIV Sexual transmission of HIV can occur through unprotected vaginal or anal intercourse, and infection appears to be facilitated by epithelial damage (104). However, studies of SIV transmission in monkeys have established that infection may also occur via either rectal or vaginal mucosae without damage of epithelial linings (105, 106). Therefore, HIV must be able to cross both simple and stratified epithelia. Free HIV as well as virus-infected cells are present in infected human semen (107). Although HIV infection of cultured human epithelial cell lines has been documented (108, 109), there is no clear evidence that normal intestinal enterocytes or vaginal epithelial cells themselves become infected in vivo (110). In human rectum, the presense of M cells over abundant rectal lymphoid follicles is well documented. The possibility that HIV could enter the human rectal mucosa via M cell transport would be greatly increased if the virus were able to adhere to M cell apical membranes. Live, infectious HIV-1

has been shown to adhere to rabbit and mouse M cells in explants of Peyer's patch mucosa, and virus was transported into the M cell pocket (111). Although similar studies have not been performed with human mucosa, it seems prudent to assume that M cell transport of HIV could occur in human rectum and that delivery of virus to the M cell intraepithelial pocket could be a rapid and efficient mechanism for infection of the $CD4^+$ T cells, macrophages, and dendritic cells that lie within and beneath the epithelium. No M cells have been identified in the vagina and uterus of nonhuman primates or women. However, the stratified vaginal epithelium contains dendritic cells that can be infected, can carry virus to draining lymph nodes and more distant lymphoid organs, and can transfer infection to T cells (31–33). Whether the subepithelial and follicular dendritic cells in mucosal-associated lymphoid tissues play a similar role in HIV infection of rectal mucosa remains to be seen. Mucosal vaccine strategies that elicit secretory antibodies may be an important component of immune protection against sexual transmission of HIV (112). Mucosal anti-HIV vaccines must be designed to interact with local M cells or dendritic cells to achieve an appropriate regional distribution of specific IgA plasma cells and IgA secretion in rectum and cervix.

Bacteria

A wide range of gram-negative bacteria bind selectively to M cells (76). Certain of these, including *Vibrio cholerae* (113, 114) and some strains of *E. coli* (115), are not normally invasive, and M cell transport leads to antigen processing and an immune response but not to mucosal disease. Others including *Salmonella typhi* and *S. typhimurium* (116, 117), *Shigella flexneri* (118), *Yersinia enterocolitica* and *pseudotuberculosis* (119, 120), and *Campylobacter jejuni* (121) exploit M cell transport to infect mucosal tissues or to spread systemically, or both.

ADHERENCE MECHANISMS The attachment of bacteria to eukaryotic cells often requires recognition of oligosaccharides containing specific sugar linkages, as evidenced by the fact that oligosaccharides are typically the strongest inhibitors of bacterial/eukaryotic cell interactions (122, 123). It is reasonable to postulate that carbohydrate-specific mechanisms would also permit certain bacteria to recognize M cells. The demonstrated diversity of M cell surface glycoconjugates may thus modulate the ability of M cells to sample microorganisms, allowing M cells to contend with the diversity of organisms in the gut lumen (51). It would also reduce the likelihood that all M cells are targeted by a given pathogen and increase the probability that the epithelial barrier is maintained during an infection. This would be particularly critical for protection from pathogens such as Salmonella that cause cytopathic effects on M cells.

It is likely that adherence and uptake of all pathogenic bacteria by M cells involves a sequence of molecular interactions including initial recognition of

M cell surface oligosaccharides by bacterial adhesins, followed by more intimate associations that could require expression of additional bacterial genes, processing of M cell surface molecules, and recruitment of integral proteins of the M cell membrane to the attachment site. The ultrastructural appearance of various bacterial–M cell interaction sites suggests that a variety of molecular mechanisms may be at play and that different bacteria activate distinct intracellular signaling systems.

E. COLI Many nonpathogenic *Escherichia coli* strains found in the intestine do not selectively adhere to epithelial cell surfaces. However, certain pathogenic strains do colonize the mucosa and interact with M cells. *E. coli* strain O:124 associated with the surfaces of rabbit M cells, was taken up and was later released into the intraepithelial pocket (124). The rabbit pathogen *E. coli* RDEC-1 initially associates with peripheral components of the M cell surface. Later it induces effacement of M cell microvilli/microfolds and formation of stable, intimate adherence sites that appear as "pedestals" supported by submembrane actin assemblies (115), similar to those observed at later times on absorptive enterocytes (125). Such structures are also induced by enteropathogenic *E. coli* (EPEC). The factors responsible for mediating intimate attachment and effacement of microvilli on enterocytes are encoded by the *eae* gene, which encodes intimin, homologous to *Yersinia* invasins, and the *cfm* gene, whose product is believed to subvert host cell signaling pathways (126, 127). Adherence of RDEC-1 to M cells does not result in bacterial uptake, perhaps because involvement of cytoskeletal structures in pedestal formation prevents phagocytosis. Initial M cell adherence may be pilus-mediated: When *E. coli* pili were expressed in a nonadherent strain of *Shigella flexneri*, the recombinant strain adhered preferentially to M cells but failed to induce pedestal formation. Instead it was phagocytosed and transported across M cells (128).

VIBRIO CHOLERAE *V. cholerae* form broad areas of very close interaction with M cell apical membranes that result in recruitment of M cell submembrane actin filaments and phagocytosis without damage to the M cell (76). *V. cholerae* is efficiently transported by M cells (113), but uptake by these cells does not cause disease; the bacteria are not equipped to survive in the mucosa or spread systemically. This makes *V. cholerae* an attractive candidate for mucosal vaccine strategies (129). Within the small intestine, *V. cholerae* express a group of coregulated proteins including adhesins that allow them to adhere to epithelia of the proximal small intestine, pili that stabilize colonies on mucosal surfaces, and cholera toxin that induces secretion of chloride ions from intestinal epithelial cells (130). The binding of Vibrios to M cells requires live bacteria in the growth phase (113) but does not require live host cells (131). It is not known whether

the same Vibrio adhesins that mediate intestinal colonization are involved in M cell adherence. In mice, M cell uptake induces specific IgA lymphoblasts in Peyer's patches (114, 132). In humans, this results in a protective mucosal immune response including secretion of IgA antibodies directed against both CT and the outer membrane lipopolysaccharide (LPS), which can be sufficient to prevent subsequent *V. cholerae* colonization and diarrheal disease (133).

SALMONELLA The pathogenesis of *S. typhimurium* in mice following ingestion of bacteria involves development of initial foci of infection in small intestinal Peyer's patches (134). *S. typhi* and *typhimurium* adhere rapidly and selectively to M cells but also can invade directly through the absorptive villus epithelium (135). The Salmonella adhesins involved in adherence to enterocytes or M cells have not been identified. In the intestine in vivo, Salmonella invades enterocytes through a mechanism that involves disassembly of microvilli, disruption of apical cytoskeleton, and uptake in large membrane-bound vesicles (135). Studies utilizing intestinal cell lines in vitro have shown that invasion is accompanied by activation of intracellular signaling pathways and local cytoplasmic Ca^{++} spikes that result in cytoskeletal rearrangements, membrane ruffling, and endocytosis of bacteria (136). Signal peptide-independent proteins secreted by Salmonella are involved in the cytoskeletal effects in host cells, and these are shared by other invasive enteric pathogens. The translocation machinery, the secretory proteins, and the chaperones in Salmonella are homologous to their Yersinia and Shigella counterparts (137). Salmonella is also able to activate the EGF receptor and, through phospholipases, to increase the level of leukotrienes that in turn open Ca^{++} channels (136, 138). M cells show a response to Salmonella attachment similar to that of enterocytes, presumably through similar molecular mechanisms. Within 30 minutes after injection into ligated intestinal loops, *Salmonella typhimurium* induced "ballooning" of the M cell apical surface and rapid uptake of bacteria, followed by degeneration and loss of M cells, which allowed the bacteria even easier access to the underlying mucosa (117).

The ability of *S. typhi* to adhere to M cells and to target themselves into Peyer's patches has been exploited in the use of attenuated strains as oral vaccines against typhoid fever (139). In addition, because of their efficient entry into mucosal inductive sites, multiple genetically engineered *S. typhi* and *typhimurium* strains have been generated and tested as vectors for expression of foreign antigens for mucosal immunization (129, 139). One of the challenges in this strategy has been achieving sufficient attenuation while still preserving M cell adherence and proliferation in the mucosa.

YERSINIA Yersinia is another invasive microorganism that exploits M cells to enter the intestinal mucosa and uses immune cells for systemic spread. Certain

Yersinia species that are capable of invading the intestinal mucosa cause enteritis as well as mesenteric lymphadenitis in mammals. In experimental animals, Yersinia adhere preferentially to M cells, and binding is followed by uptake and transcytosis (119, 120). Studies in our laboratory showed that uptake is accompanied by a disruption of M cell apical cytoskeleton and may be followed by sloughing of the FAE (H Amerongen, R Isberg, M Neutra, unpublished data). Yersinia, like Salmonella, can induce drastic changes in the F-actin network of cultured cells. The molecular steps involved include binding to integrins, activation of a tyrosine phosphatase (the Yop E protein), activation of a host protein kinase, and activation of small G proteins (rho and rac) involved in regulation of F actin assembly (140). Some of the secreted proteins are actively injected by Yersinia into host cells (141). Although nothing is known about the mechanism of adherence of Yersinia to M cells in vivo, the pathogenic potential of Yersinia has been related to several virulence factors. The Yersinia genes *inv* and *YadA*, whose products (invasins) are known to bind to $\beta 1$ integrins, are involved in adherence and invasion of cultured cells (142). Expression of invasins also correlates with the ability of Yersinia to proliferate in Peyer's patches in vivo (143). It is not clear whether receptors on M cells are recognized by these invasins, since integrins are generally present on basolateral but not apical membranes of epithelial cells (144). However, the possible presence of low levels of integrins on M cell apical surfaces has yet not been ruled out. The possibility remains that Yersinia invasins are multifunctional molecules that can interact with M cell surfaces via domains not involved in integrin binding (136).

SHIGELLA Shigella are facultative intracellular pathogens responsible for severe damage of the intestinal and colonic mucosa accompanied by loss of epithelial barrier function (145). The organisms infect cells by adhering to the plasma membrane, undergoing phagocytosis, and then disrupting the phagosome membrane to enter the cytoplasm. Once within the host cell cytoplasm, the bacteria proliferate, induce assembly of a "tail" of actin filaments, and are extruded in a cytoplasmic process which is phagocytosed by the neighboring cell, thus repeating the cycle of infection (145). Studies using polarized monolayers of intestinal enterocytes in vitro have shown that as long as epithelial tight junctions are intact, *Shigella flexneri* is unable to invade via the apical surfaces of enterocytes and can enter only via basolateral membranes (146). In contrast, inoculation of rabbit Peyer's patches with a pathogenic strain of *Shigella flexneri,* as well as selected nonpathogenic strains, resulted in selective binding and phagocytosis by M cells (118). In experimental shigellosis in vivo, entry of Shigella via M cells occurs early, followed by local invasion of all epithelial cells via basolateral cell surfaces and disruption of the entire FAE (147). Uptake of a virulent strain into Peyer's patches resulted in ulcerations in the FAE, whereas

mucosal damage was not observed with any avirulent strains. This is consistent with the observation that mucosal ulcerations typically found in Shigella infections of humans are most frequent in the colon and ileum, sites where lymphoid follicles and M cells are relatively numerous. General invasion of the intestinal epithelium is a later event and requires disruption of epithelial tight junctions (147). Shigella challenge studies using cocultures of human enterocyte monolayers and neutrophils have demonstrated that epithelial disruption is mediated by cytokines from inflammatory cells (in particular, neutrophils) that invade the epithelium in response to chemoattractants produced by Shigella (148).

CONCLUSION

All types of epithelia are engaged in providing samples of the external environment to the immune system, while maintaining a selective barrier. They accomplish this either through collaboration with professional antigen-presenting dendritic cells, or by producing a specialized epithelial phenotype, the M cell. Transport by M cells is now widely recognized as an important factor in inducing mucosal immune responses, and in the invasion of the intestinal mucosa by pathogenic microorganisms. The importance of intraepithelial dendritic cells in these phenomena is also receiving increased attention. However, much remains to be learned about the specific molecular recognition systems and nonspecific adherence mechanisms that determine the efficiency of these transport pathways. Research in this area would be greatly facilitated by development of in vitro co-culture systems that replicate the FAE with differentiated M cells, and stratified epithelia with intraepithelial dendritic cells. Information on the interactions of microorganisms with these transepithelial transport mechanisms could lead to more effective targeting of nonliving vaccines and live microbial vectors containing recombinant proteins or genes to the mucosal immune system.

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REGULATION OF MHC CLASS II GENES: Lessons from a Disease

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KEY WORDS: MHC class II deficiency, bare lymphocyte syndrome (BLS), gene regulation, RFX, CIITA, antigen presentation

ABSTRACT

Precise regulation of major histocompatibility complex class II (MHC-II) gene expression plays a crucial role in the control of the immune response. A major breakthrough in the elucidation of the molecular mechanisms involved in MHC-II regulation has recently come from the study of patients that suffer from a primary immunodeficiency resulting from regulatory defects in MHC-II expression. A genetic complementation cloning approach has led to the isolation of CIITA and RFX5, two essential MHC-II gene transactivators. CIITA and RFX5 are mutated in these patients, and the wild-type genes are capable of correcting their defect in MHC-II expression. The identification of these regulatory factors has furthered our understanding of the molecular mechanisms that regulate MHC-II genes. CIITA was found to be a non-DNA binding transactivator that functions as a molecular switch controlling both constitutive and inducible MHC-II expression. The finding that RFX5 is a subunit of the nuclear RFX-complex has confirmed that a deficiency in the binding of this complex is indeed the molecular basis for MHC-II deficiency in the majority of patients. Furthermore, the study of RFX has demonstrated that MHC-II promoter activity is dependent on the binding of higher-order complexes that are formed by highly specific cooperative binding interactions between certain MHC-II promoter-binding proteins. Two of these proteins belong to families of which the other members, although capable of binding to the same DNA motifs, are probably not directly involved in the control of MHC-II expression. Finally, the facts that CIITA and RFX5 are both essential

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and highly specific for MHC-II genes make possible novel strategies designed to achieve immunomodulation via transcriptional intervention.

FUNCTIONAL IMPORTANCE OF MHC CLASS II REGULATION

Function of MHC Class II Molecules

MHC class II (MHC-II) genes were first described as immune response (Ir) genes (1) constituting the genetic basis for high and low immunological responsiveness to certain antigens. Since the first cloning of MHC-II genes in humans, a great deal has been learned about the diversity and function of MHC-II molecules. They are heterodimeric transmembrane glycoproteins consisting of α and β chains. The different MHC-II isotypes (HLA-DR, -DQ and -DP in humans; IE and IA in mice) are encoded by distinct α chain (A) and β chain (B) genes (2). In addition to the existence of these multiple genes, the genetic diversity of the MHC-II system is characterized by an extensive degree of allelic polymorphism. More than 100 alleles for example are now recognized at the HLA-DRB1 locus.

MHC-II molecules are responsible for antigen-specific activation of $CD4^+$ T helper lymphocytes. MHC-II heterodimers are transported, in association with the invariant (Ii) chain, to a specific endocytic compartment where their polymorphic peptide binding groove is loaded with peptides derived from processed exogenous protein antigens (3). The products of the nonclassical HLA-DMA and -DMB class II genes are essential for this peptide loading step (4, 5). The MHC-II peptide complex is then transported to the cell surface where its recognition by the TCR of $CD4^+$ T lymphocytes leads to allele- and peptide-specific T cell activation. Activation of T cells can also be induced by their recognition of MHC-II molecules associated with superantigens (6, 7). In addition to their role in T cell activation, MHC-II molecules mediate trans-membrane signaling that elicits various activation processes within the MHC-II positive cell (8). This can be triggered by interaction with the TCR, superantigens, and probably other unknown ligands.

MHC Class II Regulation and the Control of the Immune Response

Expression of MHC-II genes is very tightly regulated. In contrast to the ubiquitous expression of MHC class I (MHC-I) molecules, MHC-II molecules are generally expressed on only a small number of specialized cells. Two distinct modes of expression are recognized; certain cells express MHC-II genes

constitutively; in other MHC-II negative cell types, their expression can be induced. Constitutive MHC-II expression is largely restricted to professional antigen presenting cells (APC), including B lymphocytes, dendritic cells such as Langerhans cells, and various other cells of the monocyte-macrophage lineage. Under most normal conditions, the different MHC-II A and B genes are all regulated in a coordinated fashion.

Given the essential function of MHC-II molecules in T cell activation (9), the molecular mechanisms regulating their complex expression profile obviously represent an important parameter in the normal physiological control of the immune response. In addition, defects in the regulation of MHC-II expression can have profound immunopathological consequences: A lack of MHC-II expression is known to result in severe immunodeficiency (10, 11), and ectopic or pathological upregulation of MHC-II expression (12) has been suggested to lead to the aberrant T cell activation observed in certain autoimmune diseases (13, 14). Consequently, the elucidation of the biochemical and genetic mechanisms that control the regulation of MHC-II gene expression represents a major challenge in molecular immunology and immunopathology.

Modulation of MHC Class II Expression

Expression of MHC-II molecules can be fine-tuned by a large number of different stimuli. A detailed discussion of the agents known to modulate MHC-II expression has been reviewed elsewhere (15, 16). A concise summary is difficult to make because different cell types exhibit different patterns of responses, and these responses can be strongly influenced by parameters such as the concentration, sequence of addition, and timing of exposure to the modulators, the stage of differentiation and maturation of the target cells, and differences in the sublines of the cultured cells examined. However, the major points can be summarized as follows. (*a*) Constitutive or basal expression within MHC-II positive cell lineages is regulated as a function of developmental stage and can be further modulated by a number of stimuli, including immune and neuroendocrine mediators. For example, in B lymphocytes the most effective upregulators are IL-4, IL-10, and IL-13 (17–20), while downregulation can be mediated by prostaglandins and glucocorticoids (15, 16). GM-CSF is a powerful inducer in dendritic cells (21, 22). (*b*) MHC-II expression can be induced or enhanced by IFN-γ in a variety of cell types, including cells of the macrophage-monocyte lineage, endothelial cells, epithelial cells, fibroblasts, and muscle cells. IFN-γ-induced MHC-II expression can in turn be upregulated (IL-4, TNF-α) or downregulated (TGF-β, CSF-1, IFN-α and -β) by other stimuli (15, 16, 23). The same modulator can have opposite effects in different target cells. For example, IL-10 upregulates MHC-II expression in B lymphocytes (19) but inhibits basal and IFN-γ induced expression in monocytes

(24). (*c*) An emerging concept is that modulation of MHC-II expression can also be mediated by direct cell-to-cell contact. For instance, CD40-CD40L and CD5-CD72 interactions upregulate MHC-II expression in B lymphocytes and dendritic cells (22, 25, 26), suggesting that this may occur via contact with T cells, and MHC-II expression on endothelial cells can be induced by contact with NK cells (27).

THE MHC CLASS II PROMOTER

Cis-Acting Elements

Expression of MHC-II genes is regulated primarily at the level of transcription. The main control element regulating transcription is a highly conserved 150 bp promoter proximal region situated upstream of the transcription initiation site. Although additional promoter distal regulatory elements have also been described in certain MHC-II genes (28–32), the promoter proximal region is sufficient to confer both constitutive and inducible expression in transient transfection experiments (15, 33, 34). This promoter proximal region contains sequence motifs called the S, X, X2, and Y boxes (Figure 1). Their sequences, orientations, relative positions, and spacing are conserved in all of the α and β chain genes in all species that have been examined (15, 33, 34, and references therein), including the recently isolated zebra fish MHC-II genes (35, 36). The same promoter structure is also conserved in genes known to be coregulated with MHC-II, namely the HLA-DM genes (37) and the invariant (Ii) chain gene (38, 39). The 7-bp S box (also called the H box) is situated within a 30-bp region referred to as W or Z (Figure 1). The Y box contains an inverted CCAAT sequence (Figure 1). Depending on the gene, the X2 box resembles either a cAMP responsive element (CRE) or a TPA responsive element (TRE) (Figure 1). The X box has recently been redefined as an imperfect inverted repeat related to a *cis*-acting element (EF-C or MDBP site) found in several cellular genes and viral enhancers (40, 41). The most conserved region of the X box corresponds to one half-site of the EF-C/MDBP palindrome, while the second half-site lies within a pyrimidine-rich region situated adjacent to the 5′ end of the X box (Figure 1).

The functional role of the S, X, X2, and Y boxes has been established by a large number of transient transfection experiments (see references in 15, 33, 34) and, to a lesser extent, with the use of transgenic mice (42, 43) and in vitro transcription experiments (44–49). In addition, several studies have addressed the possibility that naturally occurring polymorphisms within these *cis*-acting elements may account for allelic differences in the expression levels of individual MHC-II genes (50, 51). Although the relative contributions of

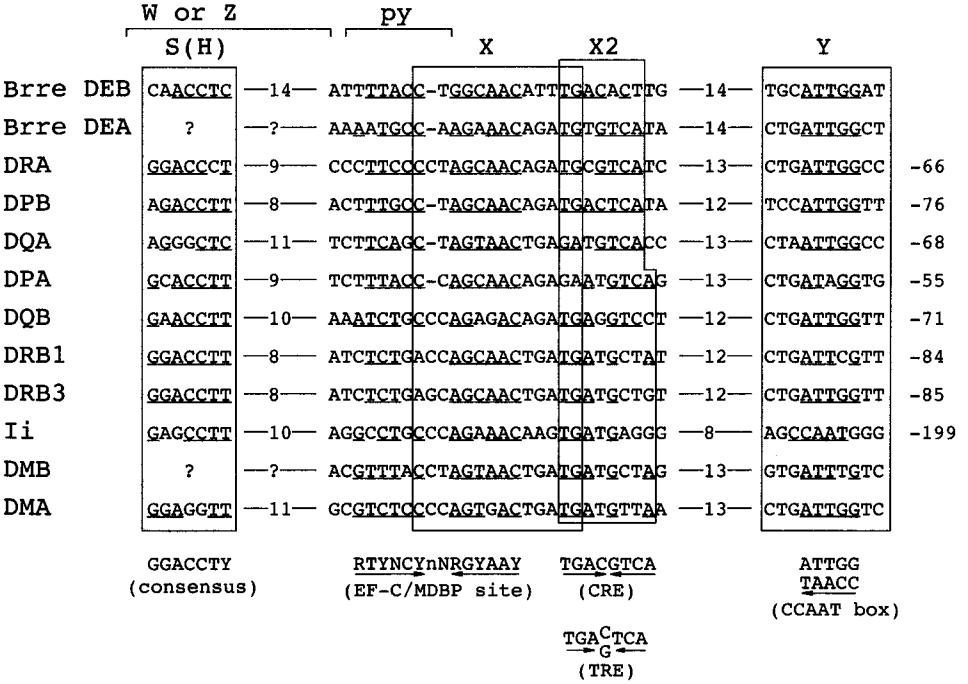

Figure 1 Sequence alignment of the conserved S, X, X2, and Y box *cis*-acting elements present in all MHC-II promoters. The sequences shown are from the human *HLA-DRA, -DRB1, -DRB3, -DQA, -DQB, -DPA,* and *-DPB* genes, from the zebra fish (Brre) *DEB* and *DEA* genes, and from the human *Ii, DMA,* and *DMB* genes. The S box, also called H, is found within a 30-bp region also referred to as W or Z. There is a pyrimidine-rich region (py) immediately upstream of the X box. Consensus sequences for the S box, the EF-C/MDBP site overlapping the py region and X box, the TRE and CRE elements corresponding to the X2 box, and the inverted CCAAT present in the Y box are indicated below. Nucleotides matching these consensus sequences are underlined. The orientation of the Y box in the Ii gene promoter is inverted with respect to the Y boxes of the other genes. Numbers indicate the distance in bp between the sequences shown, and (where it is known) the position upstream of the transcription initiation site.

the different *cis*-acting elements vary from one system to another, a remarkably consistent and coherent picture has emerged; irrespective of the system analyzed (human or mouse, MHC-II gene, cell type, constitutive and inducible modes of expression, modulation by various stimuli), the MHC-II promoter behaves as a single functional unit in which the S, X, X2, and Y boxes all contribute to optimal promoter activity. This is further emphasized by observations indicating that the individual *cis*-acting elements are functionally dependent on each other. First, the highly conserved spacing and stereospecific alignment between the S, X, X2, and Y boxes are essential for promoter activity (52–54). Second, the different MHC-II promoter elements have little activity on their own, even when multimerized, but they activate transcription synergistically when combined (see, for example, 49, 55). Finally, in vivo occupation of the X, X2, and Y boxes by their cognate DNA binding proteins appears to be controlled in a coordinated manner; all three boxes are occupied in MHC-II positive B cells and IFN-γ-induced cells, but unoccupied in uninduced MHC-II negative cells (39, 56–59).

In addition to the basic regulatory module constituted by the S, X, X2, and Y boxes, a number of *cis*-acting elements present only in certain MHC-II promoters, particularly the DRA promoter, have been described (47, 58–64). Given the fact that MHC-II genes are in general coregulated via the shared S, X, X2, and Y boxes, it is likely that such gene-specific elements play only an accessory role, for instance in enhancing the constitutive level of DRA expression in B cells.

DNA Binding Proteins

In the hope of isolating *trans*-acting factors controlling transcription of MHC-II genes, a large number of site-specific DNA binding proteins have been identified by in vitro binding assays and/or cloned by either affinity purification or screening of λgt11 cDNA expression libraries (Table 1). These approaches are based solely on the affinity of a DNA binding protein for a specific target sequence. For a number of reasons, this does not guarantee the identification and isolation of functionally relevant regulatory factors. First, the DNA fragments used may fortuitously contain potential protein binding sites that in fact do not function as *cis*-acting regulatory elements in vivo. Second, DNA binding proteins frequently belong to large families of related proteins that exhibit identical or similar target site specificities in vitro, yet have different functions in vivo. Finally, individual transcription factors do not necessarily require high affinity target sites within the regulatory regions that they control because their binding can be stabilized by cooperative interactions with other proteins binding to adjacent sites. It is therefore unlikely that all of the DNA binding proteins identified and cloned on the basis of their affinity for the S, X, X2, and Y boxes

are actually MHC-II gene regulatory factors. In fact, evidence supporting a functional role for many of these proteins is weak or indirect (Table 1). In several instances, evidence for a functional role relies exclusively on a correlation between the expression of the factor and the expression of MHC-II genes, or between the effect of promoter mutations on the activity of the promoter and the affinity of the factor for its binding site [Table 1; NF-X (45), NF-X2 (65, 66), NF-J (67), IFNEX (68–70), TIC-X (68–70), HB16 (71)]. For other factors, additional functional evidence relies on antisense experiments [Table 1; RFX1 (72), hXBP1 (73), c-Fos (74), clone 18 (75)] or on overexpression of the factor in transfected cells [Table 1; YB-1(76), NF-X1 (77)].

Both types of experiment may lead to erroneous conclusions, particularly when dealing with proteins belonging to families of related factors; overexpression of a DNA binding protein may for example cause it to bind to and sequester another crucial regulatory factor or may force an interaction with the promoter that would normally not occur at physiological concentrations. Antisense experiments are difficult to interpret because the effect on promoter activity could be indirect. It could for example result from an inhibition of the synthesis of another highly homologous *trans*-acting factor. Consequently, for many of the proteins, definitive conclusions concerning a functional role in transcription of MHC-II genes will have to await the results of genetic approaches such as knockout experiments.

Three of the MHC-II promoter binding complexes, RFX, NF-Y, and X2BP, are particularly relevant to the regulation of MHC-II genes (Table 1 and Figure 2). (*a*) RFX is a ubiquitously expressed heteromeric nuclear complex that binds to the X box of MHC-II promoters (78). Direct functional evidence for a crucial role of RFX in MHC-II gene expression has been provided by in vitro transcription experiments and by the elucidation of the molecular defect affecting certain MHC-II regulatory mutants (see below). RFX belongs to a novel family of related DNA binding proteins of which the other members (Table 1; RFX1-RFX4) (41, 79) are probably not involved in the control of MHC-II gene transcription. (*b*) The ubiquitously expressed Y box binding complex NF-Y (also called YEBP or CBF) is a heteromeric CCAAT binding protein of which three subunits have been cloned in various species (80, 81). In vitro transcription experiments have provided strong evidence that NF-Y is a transcription factor for multiple cellular genes, including MHC-II genes (48, 82, 83). In addition, NF-Y binds cooperatively with RFX (see below) (54). (*c*) The X2 box binding complex X2BP is a heteromeric nuclear protein of which the subunits have not yet been cloned (84, 85). However, it is distinct from other cloned X2 box binding proteins belonging to the Jun/Fos and ATF/CREB families (85). Although no direct functional evidence exists, a role in MHC-II

Table 1 Nuclear complexes and cloned proteins binding to the MHC class II promoter

X box	Promoters[a]	Characteristic features and comments	Suggested function[b]	References
RFX (X-A)[c]	DRA, DPA DRB1, DRB3 DQA, Eα	Nuclear complex, ubiquitous, absent in MHC-II regulatory mutants, at least two subunits (p75 and p36), p75 is RFX5, binds cooperatively with NF-Y and X2BP to all MHC-II promoters tested, essential activator of MHC-II, DM and Ii	ACT: IVT, BLS, BSM	49, 54, 55, 78, 85, 101, 102, 103, 109
RFX5	DRA	Cloned, fifth member of the RFX family, is the p75 subunit of the RFX complex, ubiquitous, mutated in all BLS patients from group C, essential activator of MHC-II, DM and Ii	ACT: BLS	109
NF-X (NF-Xc)[c]	Eα, Eβ, Aα Aβ, DRA	Nuclear complex, ubiquitous, probably represents the cloned RFX1-3 proteins	ACT: BSM	41, 45, 66, 132
RFX1-4	DRA, DPA DQA, Eα	Cloned, family of proteins with characteristic DNA binding and dimerization domains, bind as homo- or heterodimers to inverted repeats known as EF-C or MDBP sites, RFX1-3 probably correspond to NF-X	ACT: AS and BSM for RFX1	40, 41, 72, 79, 132
TRAX1	DRA	Nuclear complex, distinct from RFX and NF-X	ACT: IVT	133
NF-X1	DQB, DPB, DRA, DPA DRB, DQA	Cloned, cysteine rich DNA binding protein, enhanced by IFN-γ after a long time lag	REP: CE, TF	77
Y box	**Promoters[a]**	**Characteristic features and comments**	**Suggested function[b]**	**References**
NF-Y (YEBP, CBF)[c]	All tested	Ubiquitous, CCAAT binding factor, binds cooperatively with RFX, activator for numerous genes, including MHC-II genes, three subunits are cloned	ACT: IVT, BSM, DN	48, 54, 80, 81, 82, 83
YB-1	DRA, DQB DRB, Ii	Cloned, member of a large multigene family, low binding specificity	REP: TF, CE	76, 134

(*Continued*)

Table 1 (*Continued*)

X2 box	Promoters[a]	Characteristic features and comments	Suggested function[b]	References
X2BP (X2bp)[c]	DRA, DPB, DRB1, DRB3	Nuclear complex, ubiquitous, binds cooperatively with RFX To all promoters tested, distinct from CREB-1, CREB-2, CREM-1, ATF-2, ATF-3, c-Fos, c-Jun, hXB1	ACT: BSM	55, 84, 85, 135
NF-X2	DRA	Nuclear complex, probably corresponds to AP1 (c-Jun/c-Fos)	REP: CE, BSM	65, 66
IFNEX	DRA, RT1Bα	Nuclear complex, induced by IFN-γ only in primary astrocytes and microglial cells	ACT: CE	68, 69, 70
TIC-X	DRA, RT1Bα	Nuclear complex, induced by IFN-γ+TNF-α only in primary astrocytes	ACT: CE	68, 69, 70
hXBP1	Aα, DRA DPB	Cloned, b-Zip protein, perference for TRE sites, can heterodimerize with c-Fos	ACT: AS	73, 74, 136
HB16	Aα	Cloned, b-Zip protein, perference for CRE sites, can heterodimerize with c-Jun	ACT: BSM	71
mXBP (ATF2)[c]	Aα	Cloned, b-Zip protein, perference for CRE sites, can heterodimerize with c-Jun	ACT	137
S box	**Promoters[a]**	**Characteristic features and comments**	**Suggested function[b]**	**References**
W-B1/W-B2 NF-Zc/NF-Z2	DRA	Nuclear complexes, can also interact with *X* box of DRA	ACT, BSM	66, 138
NF-J	DPA, DQB	Nuclear complex, binds to both *S* and 'J' motifs	ACT, BSM	67
Clone 18	DPA	Cloned, zinc finger protein, binds to both S and 'J' motifs	ACT, AS	75

[a]Promoters to which binding has been demonstrated.
[b]Functional evidence for a role in activation (ACT) or repression (REP) of MCH-II genes: IVT, in vitro transcription; BLS, deficient or mutated in BSL patients; BSM, effect of binding site mutations on promoter activity; AS, antisense; TF, effect of overexpression in transfected cells; CE, correlation between expression of the factor and expression of the MHC-genes; DN, dominant negative mutants.
[c]Alternative name.

gene expression is strongly suggested by the fact that, like NF-Y, X2BP binds cooperatively with RFX (see below) (55, 85).

In conclusion, a large number of site-specific DNA binding proteins interacting in vitro with the S, X, X2, and Y boxes of MHC-II promoters have been described. Although a role in controlling MHC-II gene expression can currently not be formally excluded for any of these factors, functional evidence supporting this is in many instances lacking. The two proteins (RFX and NF-Y) for which direct functional evidence does exist are both expressed ubiquitously in all cell types, irrespective of the status of MHC-II expression. Consequently, the identification and isolation of putative regulatory factors on the basis of their ability to bind in vitro to the *cis*-acting elements of MHC-II promoters has on the whole provided limited insight into the molecular mechanisms controlling cell-specific or inducible MHC-II gene transcription. Fortunately, significant progress has recently been made by a genetic approach, namely the analysis

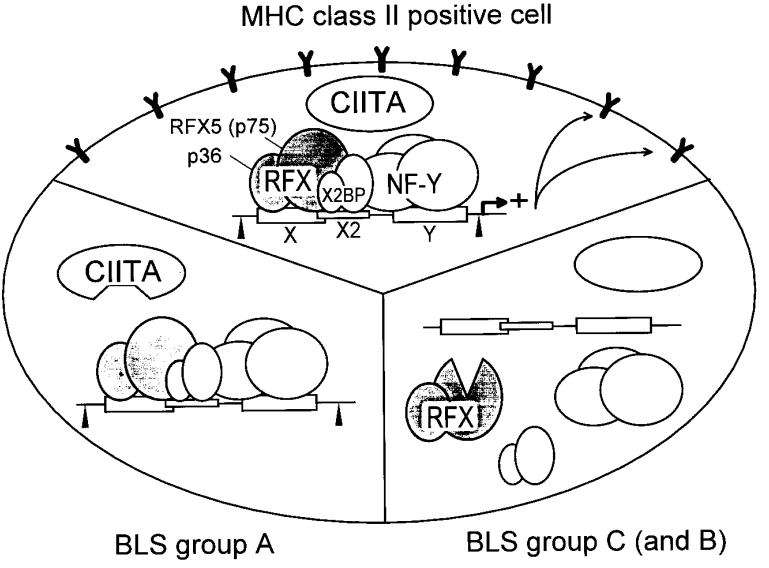

Figure 2 Two types of molecular defects in the Bare Lymphocyte Syndrome (BLS). A normal MHC-II positive cell (*top*) is compared to cells from patients in complementation groups A (*bottom left*), B and C (*bottom right*). In group A, occupation of the X, X2, and Y boxes of MHC-II promoters by RFX, X2BP, and NF-Y is normal, and the DNAseI hypersensitive sites (*arrow heads*) are present, but transcription is abolished as the result of mutations in CIITA. Patients from group B and C are characterized by a specific defect in binding of RFX. This leads to an unoccupied promoter and to the absence of the DNAse I hypersensitive sites. In group C, MHC-II deficiency is due to mutations in the RFX5 (p75) subunit of RFX.

of cell lines exhibiting a regulatory defect in MHC-II expression, particularly of cell lines derived from patients suffering from MHC class II deficiency (see below).

MHC CLASS II DEFICIENCY: A DISEASE OF GENE REGULATION

MHC Class II Deficiency or the Bare Lymphocyte Syndrome (BLS)

Severe combined immunodeficiency diseases represent a heterogeneous group of inherited disorders of the immune system. One such syndrome, MHC class II deficiency, is an autosomal recessive disease characterized by a lack of HLA class II gene expression. Less than 50 cases have been reported. A recent review of 30 patients represents the most comprehensive account of the clinical and immunological manifestations associated with the disease (10). The lack of MHC-II expression results in a severe immunodeficiency caused by an absence of both cellular and humoral immune responses to foreign antigens. There is an extreme susceptibility to viral, bacterial, fungal, and protozoal infections. Symptoms appear within the first year of life, and include primarily severe and repeated infections of the gastrointestinal and pulmonary tracts, recurrent diarrhea and failure to thrive. Death often results from persistent and multiple viral infections. The entire clinical picture can be accounted for by the absence of antigen-specific T cell activation resulting from the lack of MHC-II expression on all cells that should normally express them. The key diagnostic feature is the lack of MHC-II molecules on PBLs. There is some heterogeneity in the degree of severity of the disease, with different patients exhibiting variable residual levels of immunoglobulins and $CD4^+$ T cells. Some patients have also been reported to have a moderate reduction in the level of MHC class I molecules, but no correlation has been found either with the severity of the disease or with the genetic complementation groups to which the patients belong (10).

This form of primary deficiency has been formally named "MHC class II deficiency" by the World Health Organization (86), to distinguish it from reported examples of a selective reduction in MHC-I expression that is not associated with immunodeficiency. However, the term "Bare Lymphocyte Syndrome" (BLS), which was originally used to describe a patient who was not analyzed for MHC-II but exhibited a defect in MHC-I expression (87), is now often used synonymously for all defects involving expression of MHC-II, MHC-I, or both. This usage does not imply a common origin or physiopathology. In this review the term BLS refers exclusively to the syndrome known as MHC class II deficiency.

BLS Is a Disease of Gene Regulation

The following earlier observations were informative concerning the molecular and genetic basis for BLS. (*a*) The lack of constitutive MHC-II expression in PBL or B cell lines was observed at the levels of cell surface and intracellular MHC-II molecules (88), steady state MHC-II mRNA (88), and transcription rate of the MHC-II genes (78). (*b*) Induction of MHC-II expression by IFN-γ is totally abolished in fibroblasts from BLS patients (89). (*c*) The expression of all HLA-DR, -DQ and -DP α and β chain genes is affected (88, 90), a finding that argued against a mutation affecting only a single MHC-II gene. (*d*) In family studies, the genetic defect was found to segregate independently of the MHC (90), demonstrating that BLS is a disease of gene regulation involving genetic lesions in transacting regulatory factor(s).

When it was recognized that BLS is a disease of MHC-II gene regulation, B cell lines derived from BLS patients became extremely valuable tools for the analysis of the molecular mechanisms that control transcription of MHC-II genes. It was argued that solving the genetic basis for this disease should lead to the discovery of functionally relevant *trans*-acting factors. The first lead in this direction was the observation that the majority of BLS patients exhibit a highly specific defect in the binding activity of one of the protein complexes interacting with the promoters of MHC-II genes (78). The target sequence of this protein complex is the highly conserved X box present in all MHC-II promoters (Figures 1 and 2), and this protein complex was therefore called RFX (regulatory factor X). BLS was thus one of the first hereditary diseases in which a defect in a transacting regulatory factor was identified.

Genetic Heterogeneity in MHC Class II Regulatory Mutants

Cell fusion experiments using cell lines derived from BLS patients and a number of experimentally generated MHC-II regulatory mutants demonstrated the existence of several distinct complementation groups (Table 2) (91–94). This indicated that several different regulatory defects could give rise to a deficiency in MHC-II gene expression. The majority of BLS patients have been grouped into three distinct complementation groups (Table 2; groups A, B and C). Most of the experimentally generated mutants fall into group A. One experimentally generated mutant (95) defines a fourth complementation group (Table 2; group D) (92, 93) in which no BLS patient has yet been identified. An interesting ethnic bias is observed in the relatively small number of reported BLS patients (94). The majority of patients in group B are of North African or Turkish origin, while four of the five families in group A are Hispanic (94). Another interesting finding is that BLS patients classified in complementation groups A, B, and C exhibit an identical spectrum of clinical manifestations and

Table 2 Phenotypical, biochemical and molecular defects of MHC class II regulatory mutants from BLS complementation groups A–E

		BLS complementation groups				
	Wild type	A (II)	B (I)	C (IV)	D (III)	E
Prototypical BLS-cell line		BLS-2	BLS-1	SJO	None	ABI
Number of unrelated families		5	13	3	None	1
Prototypical in vitro mutant		RJ2.2.5	None	None	6.1.6	None
MHC class II expression	+	−	−	−	−	−
MHC class II promoter activity	+	−	−	−	−	nt
RFX-binding	+	+	−	−	−	nt
DNAse I hypersensitive sites	+	+	−	−	nt	nt
Promoter occupancy in vivo	+	+	−	−	−	nt
Genetic defect		CIITA	?	RFX5	?	?

immunopathology. The syndrome is therefore clinically homogeneous, despite the genetic heterogeneity in the cause of BLS.

Two recent reports have described BLS patients with somewhat atypical phenotypes characterized by a variable degree of residual expression of certain MHC-II genes (96, 97). One of these patients (96) seems to represent a new complementation group (Table 2, group E). One family (97) shows a considerable amount of residual MHC-II expression, and impairment of the immune response is much less severe than in classical MHC-II deficiency. The unusual phenotype in this family suggests that it may belong to a complementation group distinct from groups A to E.

A number of additional regulatory mutants have been generated by in vitro mutagenesis and selection against IFN-γ induction of MHC-II genes. Most relevant are the mutants in which the defect in IFN-γ responsiveness is specific for MHC-II genes. These include the "class A" HeLa cell mutants (98) and the "G1 to G5" series of HT1080 mutants (99). Interestingly, the latter correspond to five distinct complementation groups (99).

IDENTIFICATION OF THE GENES RESPONSIBLE FOR TWO DISTINCT MOLECULAR DEFECTS IN BLS

Evidence for Two Types of Molecular Defect in BLS

Two distinct biochemical phenotypes can be distinguished in BLS patients (11, 100) (Table 2, Figure 2). A deficiency in the binding of RFX to the X box of MHC-II promoters is detected in the majority of patients and in the experimentally generated mutant cell line 6.1.6 (49, 78, 101–103). In the case of these RFX-deficient cells, all other MHC-II promoter binding complexes are detected

normally by in vitro binding assays (49, 101–103). In the remaining patients and in a number of experimentally generated mutants, binding of all factors to the MHC-II promoter, including RFX, is normal (49, 101, 103). In vivo analysis of MHC-II promoter occupation by examination of DNAse I hypersensitive sites and in vivo footprinting also distinguishes two phenotypes. Two DNAse I hypersensitive sites flanking the MHC-II promoter in MHC-II positive cells are absent in RFX-deficient BLS cell lines (104). The altered chromatin structure revealed by these experiments is correlated with unoccupied promoters in RFX-deficient cells as assessed by in vivo footprinting (56, 57). In MHC-II regulatory mutant cell lines with normal RFX-binding, both the presence of DNAse I hypersensitive sites (P. Gönzy, W Reith, B Mach, unpublished) and in vivo promoter occupation is essentially normal (56, 57).

These two biochemical defects show a correlation with the different BLS complementation groups (Table 2, Figure 2). Cell lines from BLS complementation group A display normal RFX-binding activity and normally occupied promoters (11, 100). Cell lines from BLS complementation groups B and C show the characteristic absence of RFX-binding, DNAse I hypersensitive sites, and unoccupied MHC-II promoters.

Complementation Cloning of CIITA and Mutations in BLS Group A

A genetic complementation approach based on cDNA expression cloning was developed to identify the genes defective in BLS. The approach is based on the functional complementation of the genetic defects in MHC-II regulatory mutant cell lines by the introduction of mammalian cDNA expression libraries and selection for restored MHC-II expression (105). Optimization of a number of steps in the cloning procedure was necessary; these have been described in detail elsewhere (105, 106).

Complementation cloning was first attempted in the cell line RJ2.2.5 (107) from BLS complementation group A (91, 92). This led to the isolation of a 4.5-kb cDNA (105). Transfection of this cDNA, called CIITA for MHC class II transactivator, restores wild-type levels of HLA-DR, -DP and -DQ expression in RJ2.2.5 and all other cell lines from BLS complementation group A (105; B Lisowska-Grospierre, V Steimle, B Mach, unpublished) (Table 2). Expression of HLA-DMA and DMB is also restored (108).

Mutations in the CIITA gene have been detected in all BLS cell lines from group A so far examined (Figure 3a). In two cases, splice donor site mutations lead to exon skipping and thus to small in-frame deletions in the C-terminal portion of CIITA (Figure 3a; BLS-2 and BCH). Other defects include a point mutation (Figure 3a; BCH) and genomic deletions (Figure 3a, RJ2.2.5). All

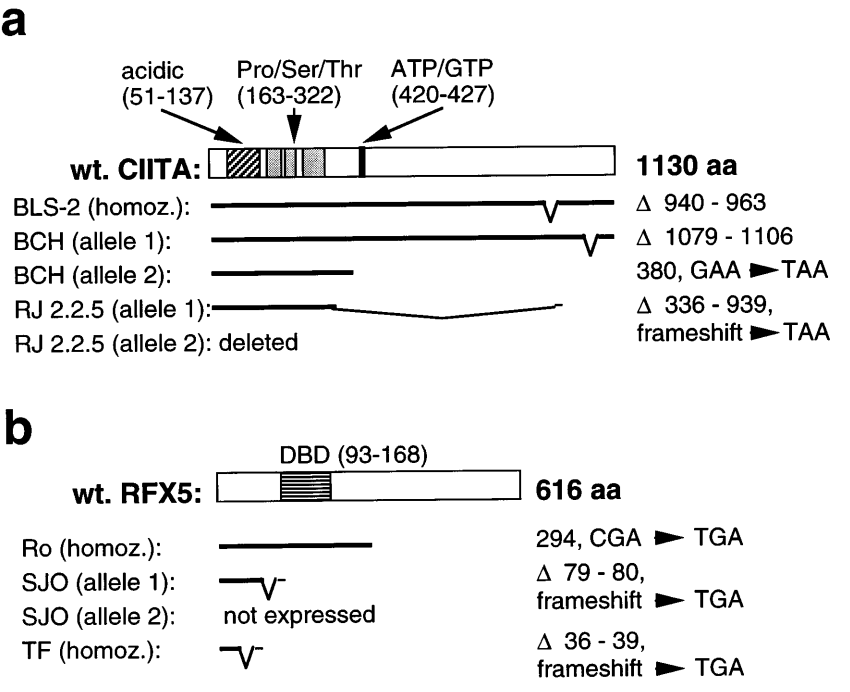

Figure 3 Mutations of CIITA and RFX5 in cells from complementation groups A and C. The repercussions of the mutations on the structure of (*a*) CIITA and (*b*) RFX5 are indicated. (*a*) Mutations in CIITA have been characterized in two BLS patients (BLS-2 and BCH) and in the experimentally generated cell line RJ2.2.5 from complementation group A. The ATP/GTP-binding motif, the acidic region, and the Pro/Ser/Thr rich regions of CIITA are indicated. (*b*) Mutations in RFX5 have been identified in all three patients (SJO, Ro and TF) from complementation group C. The DNA binding domain of RFX5 (DBD) is indicated.

mutated CIITA-alleles were shown to be inactive in transfection experiments (105; S Bontron, V Steimle, B Mach, unpublished results). Mutations in the CIITA gene are therefore responsible for the lack of MHC-II expression in BLS complementation group A (Table 2, Figure 2). Both the human and murine CIITA genes are located on chromosome 16 (V Steimle, L Otten, M Haas, J Lieman, DC Ward, B Mach, unpublished).

Cloning of RFX5 and Mutations in BLS Complementation Group C

The same complementation strategy used to isolate CIITA was subsequently applied to BLS cell lines exhibiting a defect in binding of RFX. Successful

complementation of the cell line SJO led to the isolation of a 3.4-kb cDNA capable of restoring MHC-II expression in all cell lines from group C (109). Sequence analysis revealed a homology with the DNA binding domain (DBD) characteristic of the family of X-box binding proteins RFX1–4 (41). This new family member was therefore named RFX5. Transfection of RFX5 restores expression of all MHC-II genes and the HLA-DMA and -DMB genes. Mutations in RFX5 have been identified in all three BLS patients from complementation group C, demonstrating that mutations in the RFX5 gene are responsible for this form of BLS. The mutations identified include a point mutation generating a premature stop codon (Figure 3b; Ro) and splice site mutations that lead to the use of cryptic splice sites, and hence to frameshifts and premature stop codons (Figure 3b; SJO and TF), (109; W Reith, J Villard, P van den Elsen, B Mach, unpublished). The RFX5 gene is located on human chromosome 1 (W Reith, M Morris, A Gos, B Mach, unpublished). RFX5 is a subunit of the RFX-complex found to be defective in BLS patients from complementation groups B and C (Figure 2; see below) (49, 78, 109).

ROLE OF CIITA AS A GENERAL CONTROLLER OF MHC CLASS II EXPRESSION

Mode of Action of CIITA

The mode of action of CIITA in the activation of MHC-II transcription has not yet been elucidated, but there are a number of indications that CIITA might function as a non DNA-binding coactivator. No significant homology between the 1130 amino acid sequence of CIITA and existing data base sequences has been detected. The only protein motif found in CIITA is a consensus sequence for a "P-loop" ATP/GTP binding cassette (105). No potential DNA-binding motif is evident, and no binding of CIITA to the MHC-II promoter has been observed (105). Yet it is clear, both from earlier experiments with CIITA-deficient mutants (15, 33, and references therein) and cotransfection experiments with CIITA and DRA-CAT constructs (110), that CIITA acts via the proximal MHC-II promoter. This led us to propose that CIITA might act in a coactivator-like fashion (105). The N-terminal moiety of CIITA contains regions reminiscent of transcriptional activation domains, notably a region rich in acidic amino acids and three regions having a high Pro, Ser, and Thr content. The acidic domain of CIITA is essential for its function and indeed has strong *trans*-activating activity in yeast and mammalian cells when it is coupled to a heterologous DNA-binding domain (111, 112). Furthermore, a chimeric CIITA construct, in which the acidic and Pro/Ser/Thr domains of CIITA have been replaced by a viral activation domain, retains the capacity to activate MHC-II

transcription (112). This is in agreement with our own finding that a hybrid CIITA molecule, in which the acidic region has been replaced with an acidic viral activation domain, strongly activates endogenous MHC-II transcription (V Steimle, B Mach, unpublished). Replacement of the acidic region and most of the Pro/Ser/Thr region leads to only partial activation. These findings argue in favor of a role of CIITA as a non DNA-binding coactivator, with its acidic region (and possibly also the Pro/Ser/Thr region) acting as transcription activation domains. MHC-II specificity of CIITA must reside in the remaining two thirds of the protein (112).

Interestingly, a positive quantitative correlation exists between the level of expression of CIITA and that of MHC-II. This has been observed in a large number of human and mouse tissues by quantitative RNA analysis (L Otten, V Steimle, A Mottet, B Mach, unpublished; and PF Halloran, personal communication). In addition, overexpression of recombinant CIITA by a factor of 5–10 above wild-type levels in transfected cells is accompanied by a corresponding increase in MHC-II expression (L Otten, V Steimle, A Mottet, B Mach, unpublished). It appears therefore that the level of CIITA protein determines MHC-II promoter activity.

Essential Role of CIITA in MHC Class II Modulation

In addition to its essential role in constitutive MHC-II expression in B cells, CIITA also plays a key role in the modulation of MHC-II expression in other cell types. This can be deduced from four observations (110). (*a*) CIITA is itself differentially expressed, and CIITA expression can only be detected in MHC-II positive cell lines and tissues (105) (A Mottet, L Otten, V Steimle, B Mach, unpublished). (*b*) CIITA expression is induced by IFN-γ, and this induction precedes MHC-II mRNA induction by several hours. (*c*) Fibroblasts from CIITA-deficient BLS patients cannot be induced by IFN-γ to express MHC-II molecules. (*d*) Constitutive expression of recombinant CIITA renders MHC-II negative cell lines MHC-II positive in the absence of IFN-γ (110).

Taken together, these observations clearly demonstrate that CIITA-expression is both necessary and sufficient for the activation of MHC-II gene transcription in IFN-γ-inducible cells and that IFN-γ-induced MHC-II expression is mediated via the induction of CIITA expression (Figure 4). Two subsequent studies arrived at similar conclusions (113, 114). Interestingly, the experimentally generated mutant cell line G3A (99) shows a defect in the induction of CIITA mRNA (113).

Like all other IFN-γ inducible genes, CIITA cannot be induced in Jak-1-deficient cell lines (114). The characteristics of CIITA-induction by IFN-γ are very similar to those of the guanylate binding protein (GBP) gene (110). Both

behave as IFN-γ-inducible genes of the "intermediary" type (110). However, from the study of IRF-1 knockout mice (115), a difference between the induction of CIITA and GBP can be predicted. In IRF-1$^{o/o}$ mice, MHC-II induction is normal while GBP cannot be induced (115), indicating that CIITA induction is mediated via an IRF-1 independent pathway.

CIITA also plays a key role in the extinction of MHC-II gene expression in plasma cells (116), a process thought to be due to dominant repression mechanisms (117). The lack of MHC-II transcription in plasmocytoma cells is correlated with unoccupied MHC-II promoters and undetectable CIITA expression. Expression of CIITA following transfection is sufficient to induce promoter occupation and MHC-II expression in these cells (116). It has also been shown that lack of expression of MHC-II in mouse T lymphocytes is due to lack of CIITA expression (117a).

These observations indicate that the control of MHC-II expression is regulated in many, perhaps all, biological situations via the control of CIITA expression. CIITA is thus a "master control factor" for MHC-II expression

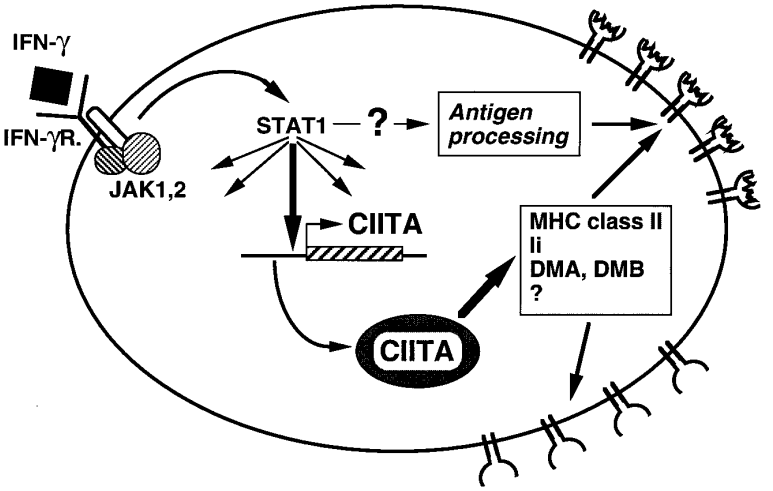

Figure 4 Schematic representation of the role of CIITA in the modulation of MHC-II expression. Activation of the interferon γ receptor (IFN-γR) generates intracellular signals (activation of Jak kinases and STAT proteins) that induce expression of the CIITA gene. CIITA then activates transcription of the MHC-II, Ii, and DM genes. Antigen processing and presentation by MHC-II molecules in IFN-γ-treated cells requires an additional IFN-γ-induced function that is not under the control of CIITA. (Jak: Janus kinase; STAT: signal transducer and activator of transcription.)

(110). This obviously refocuses the interest in MHC-II regulation toward the regulation of CIITA itself. Interestingly, the CIITA gene is controlled by at least four independent promoters, two of which account for most of its expression. It is of particular interest that one promoter primarily controls constitutive expression, while another is largely responsible for IFN-γ inducible expression (A Mottet, L Otten, V Steimle, B Mach, unpublished). Work is in progress to determine which of the different CIITA promoters are implicated in modulation of MHC-II expression by other stimuli, such as induction by contact with NK cells (27) and inhibition of IFN-γ induction by IFN-β and TGF-β (15, 16).

Role of CIITA in Antigen Processing and Presentation

CIITA also controls the expression of the invariant (Ii) chain, HLA-DMA and -DMB genes involved in antigen processing and presentation (108, 118). It has been postulated that expression of MHC-II, Ii, and DM molecules is sufficient to generate an "operational" MHC-II peptide loading compartment in nonprofessional APCs (119). Since all of these molecules are expressed under the control of CIITA, cells transfected with CIITA would thus be expected to behave as efficient APC. Unexpectedly however, CIITA-transfected melanoma cells, although expressing MHC-II as well as DM and the invariant chain, exhibit a novel antigen-processing defect (120). While they are highly efficient in the MHC-II-restricted presentation of exogenously added peptide antigens to T lymphocytes, they are deficient in the presentation of protein antigens, indicating an antigen-processing defect. This defect can be corrected by treatment of the transfectants with IFN-γ. Consequently, efficient processing and presentation of protein antigens in nonprofessional APC require not only MHC-II, DM, and Ii, but also an additional IFN-γ-inducible function that is not under the control of CIITA (Figure 4) (120).

FUNCTION OF RFX

RFX Is an Essential MHC Class II Transcription Factor

The native RFX complex has been purified to near homogeneity by affinity chromatography and shown to be a heteromeric complex consisting of at least two different subunits, p36 and p75 (49). Cross-linking experiments have suggested that both of these subunits contact the X box target site (49). In BLS patients from complementation groups B and C (49, 78, 101, 103), as well as in the experimentally generated mutants 6.1.6 (102) and one of the IFN-γ response mutants (113), binding activity of the heteromeric RFX complex is not detected, suggesting that RFX is essential for MHC-II expression. Direct evidence for this was provided by in vitro transcription experiments; the MHC-II promoter

is transcriptionally silent in nuclear extracts derived from RFX-deficient BLS cells in groups B and C, but wild-type activity is restored by supplementing these extracts with affinity purified RFX (49).

RFX5, the gene mutated in complementation group C, encodes a protein that contains the DNA binding domain typical of other known X box binding proteins (Table 1; RFX1–RFX4) (41). This suggested that RFX5 was likely to be a subunit of the RFX complex. Several independent lines of evidence confirmed this (109). First, the DNA binding domain of RFX5 exhibits specific binding to the X box. Second, binding of RFX is restored in BLS cells from complementation group C by transfection with RFX5. Third, RFX is recognized by antibodies specific for RFX5. Finally, peptide sequences derived from RFX have demonstrated that RFX5 is identical to p75. The demonstration that the p75 (RFX5) subunit of RFX is mutated in all BLS patients in group C validates our previous interpretation that a deficiency in RFX is indeed responsible for the lack of MHC-II expression in these patients (78).

RFX Promotes Cooperative Binding Interactions Required for Promoter Occupation

The MHC-II promoter behaves as a single functional unit in which the S, X, X2, and Y boxes all contribute to optimal activity. Taken individually, these *cis*-acting elements have little activity, but they function synergistically when combined. Moreover, they must be positioned correctly with respect to each other, such that they exhibit a precise stereospecific alignment on the double helical DNA (52–54). These considerations suggested that the activity of MHC-II promoters was likely to be dependent on cooperative protein-protein interactions between *trans*-acting factors binding to adjacent target sites. Studies designed to address this question have indeed shown that RFX binds cooperatively with the X2 box binding factor X2BP and with the Y box binding factor NF-Y to form higher order protein-DNA complexes containing RFX + X2BP, RFX + NF-Y, and RFX + NF-Y + X2BP (54, 55, 85). The interactions of RFX, X2BP, and NF-Y with their respective target sites are strongly stabilized in these higher order protein-DNA complexes (54, 55, 85). Functional relevance of these higher order complexes is demonstrated by the fact that promoter mutations that disrupt them have a strong negative effect on promoter activity (54, 55). Cooperative binding interactions between RFX, X2BP, and NF-Y are thus crucial for the function of MHC-II promoters.

In vivo footprint experiments have shown that the entire MHC-II promoter, including the X2 and Y boxes as well as the X box, is unoccupied in RFX-deficient BLS cells. Given the cooperative binding interactions observed in vitro between RFX, X2BP, and NF-Y, it now appears likely that the lack of RFX

binding activity in such BLS cells is directly responsible for the bare promoter phenotype. RFX probably functions in vivo as an "accessibility factor" that promotes stable promoter occupation by recruiting NF-Y and X2BP to the Y and X2 boxes, respectively, via cooperative protein-protein interactions.

Since all MHC-II genes are silent in RFX-deficient BLS patients, RFX must be essential for the coordinate transcription of the α and β chain genes of all three MHC-II isotypes. Previously, this appeared to contradict the fact that RFX cannot on its own bind all MHC-II promoters: by itself, RFX binds efficiently to certain promoters (e.g. DRA and DPA) but only very poorly or not at all to others (e.g. DQA, DRB1, and DRB3) (55, 121, 122). It is now clear, however, that binding of RFX can be strongly stabilized by X2BP or NF-Y. This stabilization is sufficiently strong to allow formation of the higher order RFX + X2BP and RFX + NF-Y complexes on the promoters of the DQA, DRB1, and DRB3 genes (55, 122; W Reith, unpublished data). RFX can thus be recruited via cooperative protein-protein interactions to promoters containing only very low affinity X box sites. Stabilized binding of RFX by NF-Y probably also explains the observation that destruction of the Y box leads to reduced in vivo occupation of the X box (123).

UNRESOLVED ISSUES AND PERSPECTIVES

Molecular Defect in BLS Group B and Other RFX-Deficient Mutants

Binding activity of RFX is undetectable in all BLS patients in complementation groups B and C (49, 78, 101, 103), as well as in an experimentally generated B cell mutant (6.1.6, group D) (102) and one of the MHC-II-specific IFN-γ responsive mutants (113), suggesting that defects leading to a deficiency in RFX binding are genetically heterogeneous. This was demonstrated functionally by the fact that affinity-purified RFX can complement in vitro transcription extracts derived from BLS patients with different genetic defects (cells in groups B and C) (49). In view of this genetic heterogeneity, it is of interest that RFX is a heteromeric complex containing at least two different subunits, p75 and p36, both of which probably contact the DNA (49). Since mutations in p75 abolish binding of RFX in BLS patients in group C, it is tempting to speculate that p36, or another as-yet-unidentified subunit of RFX, could be mutated in complementation group B. Alternatively, the defect could affect a cofactor or modifying activity required for binding of RFX. The same holds for the two experimentally generated RFX-deficient mutants, although it should be mentioned that complementation of in vitro transcription extracts by RFX has not been reported for these mutants.

Molecular Defects in Other MHC Class II Regulatory Mutants

Elucidation of the molecular defects affecting the new BLS patients (96, 97) may yield additional regulators of MHC-II expression. Of particular interest is the persistence of a significant level of expression of certain individual MHC-II loci (97). This differential effect of a regulatory defect on different MHC-II genes contrasts sharply with the situation observed when MHC-II deficiency is due to a defect in RFX or CIITA. It might be expected that mutations of a general and essential regulator of MHC-II expression should not lead to a locus-specific defect. However, the activity of MHC-II promoters depends on cooperative interactions between several distinct DNA binding factors. When analyzed on their own, these factors exhibit different affinities for different MHC-II promoters, and these different promoters may thus be more or less dependent on cooperative binding interactions. For example, RFX can bind on its own to the DRA promoter (78) but requires help from NF-Y and X2BP at the DRB1 promoter (55, 122; W Reith, B Mach, unpublished). Consequently, mutations that weaken the protein-protein interactions between MHC-II promoter binding proteins may affect different promoters to different extents.

MHC-II-specific, IFN-γ-unresponsive mutants should also provide valuable information. Since CIITA is the IFN-γ-induced mediator of MHC-II expression, some of these mutants may contribute to the elucidation of the mechanisms regulating expression of the CIITA gene.

Mode of Action of CIITA

Although the key function of CIITA in MHC-II expression is now well established, its mode of action remains elusive. First, it does not appear to be a DNA binding protein, yet it contains a region capable of functioning as an activation domain. This suggests that CIITA is a non-DNA binding coactivator that is presumably recruited to the MHC-II promoter via protein-protein interactions with one or more of the factors binding there. However, such interactions remain to be documented. Second, a potential role of CIITA in promoter occupation needs to be addressed because of the following paradox. The MHC-II promoter is occupied in CIITA-efficient B cells such RJ2.2.5 and BLS2 (56, 57) but is unoccupied in uninduced MHC-II negative cells such as fibroblasts and HeLa cells (58, 59), which do not express CIITA but contain normal levels of RFX, X2BP, and NF-Y. Transfection of CIITA into such cells is sufficient to induce promoter occupation (A Mottet, V Steimle, B Mach, unpublished). It thus appears that CIITA is required for promoter occupation in certain MHC-II negative cells but not in B cells. This is further complicated by the fact that in one CIITA-deficient BLS cell line, promoter occupation is only partial (57).

The explanation for this cell type–specific difference in the requirement for CIITA in promoter occupancy remains obscure.

Reduction in the Level of MHC Class I in BLS

A reduction in MHC-I expression on fresh PBL has been described in certain BLS patients (10, 124). This reduction does not correlate with one particular complementation group or with clinical manifestations of the disease (10). We have not observed this reduction on B cell lines established from BLS patients, although others have reported a slight reduction on such cells (J-L Touraine, personal communication). The reason for this reduction remains unexplained. It does not appear to be due directly to defects in RFX5 or CIITA, because transfection of these genes into BLS cells from complementation groups C and A, respectively, has no effect on the level of MHC-I expression. In addition, transfection of CIITA into various other cell types does not affect MHC-I expression. One possible explanation is that the MHC-I reduction observed in the patients is an indirect effect due to the lack of cell surface MHC-II or resulting from the deficiency in the activation of T cells and/or APCs.

Residual Presence of CD4 T Lymphocytes in the Absence of MHC Class II Expression

Positive selection driven by MHC-II is believed to be necessary for the thymic maturation of $CD4^+$ T lymphocytes. The almost complete absence of circulating $CD4^+$ T cells in MHC-II knockout mice is consistent with this (125, 126). Surprisingly, in the majority of BLS patients, the levels of circulating $CD4^+$ T cells are only mildly reduced compared to those in the MHC-II knockout mice. The $CD4^+$ T cell count varies from one patient to another, but this variation shows no correlation with the known genetic complementation groups. This difference in the $CD4^+$ T cell levels between MHC-II knockout mice and MHC-II-deficient BLS patients is reminiscent of the situation resulting from mutations in Zap-70; $CD4^+$ T cells are absent in ZAP-70 knockout mice, whereas they are present in human patients having mutations abolishing ZAP-70 activity (127). Two explanations can be proposed for the presence of $CD4^+$ T cells in BLS patients: (*a*) MHC-II regulatory defects such as mutations in CIITA or RFX5 may allow a low residual level of MHC-II expression in the thymus, and this may be sufficient for the maturation of some $CD4^+$ T cells. (*b*) Alternatively, maturation of a small number of $CD4^+$ T cells may be driven by other ligands, such as MHC-I molecules. This unorthodox pathway has been postulated to account for the few thymocytes committed to the $CD4^+$ lineage in MHC-II knockout mice (128), but the issue is controversial (129).

THERAPEUTIC PERSPECTIVES: MHC CLASS II TRANSACTIVATORS AS IMMUNOMODULATORS

Gene Therapy for BLS

The only therapy currently available for BLS is bone marrow transplantation, and, as indicated in a recent report (10, 130), the overall clinical outcome of this therapy in BLS is much worse than in certain other primary immunodeficiencies, particularly with haplo-identical donors. This is attributed to intractable persistent viral infections. As in the case of gene therapy for ADA (adenosine deaminase deficiency), restoring the wild-type gene in the patient's hematopoietic cells is a logical therapeutic strategy for BLS. Unfortunately, CIITA expression is tightly regulated, and it is unlikely that a normal expression profile can be conferred easily on CIITA transgenes. In the case of complementation groups B and C, however, the ubiquitous expression of the RFX complex indicates that transfer of the relevant wild-type genes into either multipotent precursor cells or PBLs of BLS patients should be considered seriously.

The CIITA Gene in Cancer Immunotherapy

Numerous attempts have been made to boost the endogenous immunogenicity of cancer cells, either in vivo or ex vivo, to induce immunological protection. Recent reports have stressed the role of MHC-II-mediated anti–tumour cell responses in immunotherapy (131). Transfer of the CIITA gene into tumour cells will induce MHC-II expression on these cells. This should perhaps be attempted, possibly in combination with B7 genes, as a novel approach for cancer vaccination.

MHC Class II Transactivators as Targets for Novel Immunomodulators

Intervention in the expression of endogenous genes is a promising therapeutic strategy. Contrary to most known transcription factors, both CIITA and RFX5 exhibit two properties that make them excellent potential targets in such a strategy. Both are absolutely essential and highly specific for MHC-II genes. It follows that novel inhibitors of either the synthesis or the activity of these two factors should behave as powerful immunomodulators, capable of reducing the level of MHC-II expression in a reversible manner in situations such as organ transplantation and autoimmune diseases.

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ON IMMUNOLOGICAL MEMORY

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ABSTRACT

Immunological memory is a hallmark of the immune system. Evolution can teach us which effector arms of immunological memory are biologically relevant against which virus. Antibodies appear to be the critical protective mechanism against cytopathic viruses. Since these viruses cause cell damage and disease directly, particularly in the absence of an immune response, mothers protect their offspring during a critical immunoincompetent period (a consequence of MHC-restricted T cell recognition) by passive transfer of neutralizing antibodies. In contrast, CTL appear to be the crucial effector mechanism against noncytopathic viruses. Since MHC polymorphism has made vertical transmission of T cell memory impossible, immunoincompetent offspring are not, and need not be, protected against such noncytopathic viruses. During the primary response and again during secondary infection, the most important function of CTL is to eliminate noncytopathic viruses, which may otherwise cause lethal immunopathology. Increased precursor frequencies of B and T cells appear to remain in the host independent of antigen persistence. However, in order to protect against cytopathic viruses, memory B cells have to produce antibody to maintain protective elevated levels of antibody; B cell differentiation into plasma cells is driven by persisting antigen. Similarly, to protect against infection with a noncytopathic virus, CTL have to recirculate through peripheral organs. Activation and capacity to emigrate into solid tissues as well as cytolytic effector function are also dependent upon, and driven by, persisting antigen. Because no convincing evidence is available yet of the existence of identifiable B or T cells with specialized memory characteristics, the phenotype of immunological memory correlates best with antigen-driven activation of low frequency effector T cells and plasma cells.

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INTRODUCTION

Browsing through textbooks and authoritative texts quickly reveals that the definition of immunological memory is not straightforward. All agree that immunological memory is illustrated by the fact that hosts once infected, and therefore having mounted an immune response and survived the infection, are protected more efficiently against second infections. Accordingly, a more rapid and more potent response exhibited in hosts that already have encountered the same antigen some time before is usually considered to reflect immunological memory. Whether this involves critical B cell or T cell responses, or both, is often not clearly distinguished (Table 1). The identification of essential cellular parameters needed for this memory reaction is far from complete, and whether memory as measured in vitro is relevant for protective immunological memory in vivo remains an open question.

Four classes of definitions, not mutually exclusive, have been proposed to account for memory. They use different levels within the complexities of the biology, and they are often difficult to compare: First, memory is the result of increased precursor frequencies of specific resting B or T cells, maintained in an antigen-independent fashion (1–4). Second, memory represents a special quality of specific lymphocytes situated partway between the virgin and the effector stage. According to this X-Y-Z proposal, where X are uninduced, Y are the "half-induced" memory-type T cells, and Z the fully differentiated effector T cells, the lifespan of the respective cells varies, and Y cells live considerably longer than Z cells (5). Third, memory represents elevated frequencies of activated, specific lymphocytes; the elevated frequencies are maintained by the triggering of recurrent infections involving persisting or cross-reactive antigens, resulting in an equilibrated level of induction of specific effector cells (6, 7). Fourth, memory reflects "regulatory" influences (e.g. networks).

Immunological memory has been reviewed recently by Gray (6), MacKay (8, 9), Doherty (4), Ahmed (10), Sprent (11), and earlier by others (12–14). The present review does not aim at extending these detailed analyses. We discuss memory mainly from the point of view of virus infections: How memory is selectable in evolution, i.e. how it enhances survival of the species. Considering the differing definitions of immunological memory and the possible mechanisms involved, the following questions arise: 1. What function might immunological T cell, B cell, and antibody memory together serve? 2. Is this function subject to evolutionary selection from the point of view of virus infections? 3. Are the methods used to measure memory adequate to characterize the phenomenon, or alternatively, what parameters should better be monitored? 4. What is the role of antigen in memory?

Table 1 Critical immunological effector mechanisms for protection against secondary virus infections

		Most important or limiting for protection[a]					
		CTL (perforin)		T (interleukins)			Protection of neonates
Virus		Resting	Activated	Resting	Activated	Antibody	by antibodies
Cytopathic	i.v.	−	−	−	−	++	+
	periphery	−	−	−	++	+	+/−
Non-cytopathic	i.v.	+	+	−	−	+	+ (not necessary)
	periphery (antiviral)	−	++	−	−	−	− (not necessary)
	periphery (immunopath.)	−	++	−	−	−(can enhance immunopathology)	−

[a]The combinations marked with + or ++ point out the critical memory effector mechanism.

This review concludes that elevated levels of memory antibodies are critical for survival of the species, that there exists probably no special and definable memory status of T cells, and that low-level persisting antigen drives low-level B and T cell responses that mediate memory functions.

THE ROLE OF IMMUNOLOGICAL FACTORS AND PARAMETERS OF INFECTIOUS AGENTS INFLUENCING IMMUNOLOGICAL MEMORY

Adaptive immune mechanisms protect vertebrate hosts against many life-threatening infections. There is good evidence that infectious agents have coevolved with their hosts' immune systems to reach a balanced state, i.e. immunological and viral parameters are interdependent (15–19) (Table 1). Immune effector mechanisms are the specific part of this overall evolutionary equilibrium, directing many nonspecific host parameters (including complement, interleukins, interferons, phagocytes) to eliminate the infection.

Specificity is the basis of the so-called self-nonself discrimination and of memory, but all three are usually considered hallmarks of the immune system. Although great efforts have been spent on defining specificity, this key parameter is still poorly understood. The relevance of thresholds of the binding forces of antibodies or T cells, of their affinities and/or avidities, and of the need for receptor cross-linking for protective immunological effectiveness against infectious agents is still far from clear.

The questions of what immunological memory is and how it is maintained are intimately linked to our understanding of specificity. If protective effector antibodies need to bind a neutralizing epitope of a virus with an affinity of at least 10^7 to 10^8 M^{-1} (20), the questions arise, whether antigens that cross-react

at the 10^6 to 10^7 M^{-1} level can induce a primary response or restimulate a memory response (or both, or neither) in vivo, and what the relevance of this response to survival might be (21). In addition, while the biological relevance of low-affinity antibodies is documented for opsonization of bacteria to enhance their elimination (22), the protective effectiveness of antibodies blocking life-threatening effects of viruses or toxins is not proven. For neutralization, or blocking, of viruses or toxic molecules, antibodies must compete in binding to the respective receptors. Experience with closely related but serologically distinct viruses, bacteria, or classical parasites suggests that relatively high-affinity thresholds for both induction and effector function of lymphocytes keep the system specific (23–30). However, for each effector mechanism and pathway, these thresholds differ and therefore have to be analyzed separately.

T cell specificity is not yet generally defined in terms of T cell receptor avidity for MHC-complexed peptides nor with respect to the required extent of receptor cross-linking (31–36). A correlation of in vitro measurements with in vivo protective activity is difficult to establish unequivocally. It is therefore difficult to extrapolate from in vitro measured cross-reactivity of memory T cells to protective activity in vivo and to its survival value. In conclusion, specificity of T cells, and also of immunological memory, is best defined operationally as selectable protective capacity in vivo.

Immunological parameters are strongly dependent upon the infectious agent; its localization and distribution and the kinetics of infection all play a major role in defining immunological defense and memory (16, 37). The example of the epidemiology of measles virus infections on the Faeröe Islands or on Pacific islands has, both earlier and again recently, been cited as proof for antigen independence of T cell memory (2, 3). But this example may be faulty for several reasons: 1. Antibodies (not $CD8^+$ T cells) seem to be key protective factors against a second measles virus infection, and 2. measles virus may persist as incomplete viral information for a long time (38). 3. Alternatively, antibody antigen complexes, particularly involving the relevant neutralizing determinants, probably persist on follicular dendritic cells for life, or 4. these antigens may be produced from internal usually undefined sources (see How Does Antigen Persist?) (39, 40).

Another interesting example is only rarely quoted: von Pirquet showed in 1907 (41) (in an experiment obviously not possible today) that children vaccinated against smallpox with live vaccinia virus were resistant to infection of the skin with a second vaccinia virus dose administered within 3–4 weeks; however, if revaccination was performed later, the children developed local vaccine lesions, but they were still protected from viremia. This example illustrates that vaccination may result in antibody-mediated protection against systemic spread

and therefore against dangerous disease. Antibodies do not however prevent local reinfection and peripheral disease manifestation. The latter are apparently best controlled by activated T cells (see later). Thus, infectious agents differ vastly with respect to their port of entry, local disease manifestations on mucosa or skin and in solid tissues, and how they spread (15, 16). Accordingly, different effector mechanisms are critical for their elimination (Tables 1 and 2).

Viruses replicate in living cells with consequences that permit their separation into two categories (Table 1). Cytopathic viruses cause host cell destruction directly. Death occurs if cytopathic virus cannot be stopped early before the virus has destroyed too many essential host cells. Immune protection is mediated by a blockage of the infection itself (by neutralizing antibodies) and/or of the initial steps of replication [by some antibodies (42)]. It is also blocked by cytokines such as interferon (IFN)α, β, in general, and then by interleukins released by specific T cells such as IFNγ and tumor necrosis factor (TNF); perforin-dependent cytolysis is apparently not important (43–46). Noncytopathic viruses do not cause host cell destruction directly; however, the induced cytotoxic T cell (CTL) response may cause destruction of infected essential host cells by lysis and/or by excessively produced interleukins (e.g. TNF, IFNγ) (47–53).

Since noncytopathic viruses cause no harm to cells whereas cytolytic CTL do, the question has to be answered: Why should these effector T cells have developed at all? Most probably because MHC class II molecules that are critical for most immune responses, including most long-lived memory antibody responses, are expressed on phagocytes and lymphocytes, but not on most cells of solid peripheral organs. Immune surveillance of dangerous alterations

Table 2 Protective capacity of activated versus nonactivated CTLs dependent upon frequencies

	Frequency of CD8$^+$ T cells					
	Not activated			Activated		
Protection against challenge infection	Low naive	High memory (− ag)	Very high (naive TCR tg)	Low	High memory (+ ag)	Very high memory (TCR tg) (+ ag)
VV (early after challenge)	no	not done	no	3	yes	yes (expected)
VV (late after challenge)	no	no[119]	no		yes[83]	yes[119]
VSV, influenza	no	not[1]	n.d.		no[1,2]	n.d.
LCMV (Systematic, i.v.)	no	yes[3]	yes[82]		yes[3]	yes[2]
LCMV (peripheral, i.c.)	no	no[2]	no[82]		yes[157]	yes[2]
LCMV (peripheral, i.f.)	no	no[2]	no[116]		yes[157]	yes[116]

[1]The experiments have actually not been performed or published; the expected result is given.
[2]Unpublished.
3 This phenotype does not exist.

of these latter cells by infection with non-cytopathic infectious agents or by transformation to form tumors is inducible only in lymphoid organs (see below), and therefore a mechanism for antigen release is necessary. $CD8^+$ T cells that exert two functions at the same time, i.e. cytolysis by perforin-dependent mechanism and release of interferons and inflammation-promoting interleukins, promote both antigen release and phagocytosis plus transport to local lymphatic organs.

The kinetics of noncytopathic virus infections and of CTL responses is important to the disease caused by CTL-mediated destruction of infected host cells.[1] While a fast response against a slow noncytopathic virus limits immunopathological host cell damage efficiently, a slow CTL response against a fast-replicating virus results in extensive and sometimes lethal immunopathology (56–58) (Table 1) by both lytic and interleukin-mediated mechanisms.

Let us emphasize here that all successful vaccines used in humans so far operate via neutralizing antibodies (59); even for smallpox, prevention of hematogenic spread of virus by antibody is probably the most important protective mechanism. A possible reason for the dominant role of high memory antibody is discussed in the next section. The example of immunity against vaccinia virus shows that local infection in the periphery is often not controlled by antibodies because they cannot reach the peripheral site of infection. Because peripheral infection by a cytopathic virus is usually restricted initially, this IL-T memory effector pathway is not crucial to survival, since neutralizing memory antibody responses critically prevent systemic spread and death (59).

It is important to emphasize that $CD8^+$ T cells can mediate antiviral protection via two pathways, either via perforin-dependent cytotoxicity (the role in immune protection of Fas-Fas ligand interaction is not yet understood) (60) or via released interleukin such as IFN and TNF (43, 44). Recent studies with perforin-deficient mice have revealed that perforin-dependent CTL activity is essential to the control of noncytopathic viruses such as lymphocytic choriomeningitis virus (LCMV) in mice and possibly of hepatitis B or C virus (HBV, HCV) (52) and human immunodeficiency virus (HIV) during primary infection. Perforin-dependent CTL activity also controls virus at low levels of persistence (53). Since memory antibody cannot reach infected peripheral cells and cannot neutralize intracellular virus, control of cell-to-cell spread of virus from (and/or destruction of) kidney tubular cells, cells of the salivary gland, or neurons (49–51, 61) that are persistently infected or reinfected by noncytopathic virus are an essential function of memory CTL, a function which apparently

[1]CTL is used here and throughout as an abbreviation for $CD8^+$ T cells that mediate their effector function via a perforin-dependent pathway (54, 55). In contrast $CD8^+$ T cells or $CD4^+$ T cells mediating effector functions via released/induced interleukins are called IL-T cells.

cannot be mediated by antibodies or IL-releasing T cells. Unless this control is fast, host cell destruction by CTL or induction of excessive inflammation by interleukins released by T cells causes disease and death by immunopathology (62–64). An example for interleukin-mediated lethal immunopathology is revealed by LCMV-infection of perforin-deficient mice (54). Thus, while antibodies can neutralize infectious agents in blood and on mucosa, cellular immunity acts efficiently against infectious agents in solid tissues.

WHY IMMUNOLOGICAL MEMORY? AN ATTEMPT AT AN EVOLUTIONARY EXPLANATION

Immunological memory must provide a survival advantage and must be selectable. Although immune hosts are in general fitter and therefore have a survival advantage, it can be argued that, if an immunologically competent host survived the first infection, the host will also survive a second infection. For immunoincompetent hosts during fetal life and in the neonatal period, immunological protection is, however, essential for survival. Such protection is absolutely necessary against cytopathic viruses but not against noncytopathic ones, since the latter viruses can establish a virus carrier state that is not life-threatening to the immunoincompetent host. Against cytopathic viruses, protection of immunoincompetent offspring is mediated by elevated protective antibody titers, but not by T cells, for the following reason: Only antibodies can be transmitted from mother to offspring (65, 66), either in mammals or birds. The most obvious requirement for such transfer appears to be the need of young immunoincompetent offspring for protection from otherwise lethal pathogens during the period when their immune system is still maturing.

The complicated process of T cell maturation was necessitated by the development of MHC-restricted T cell recognition. MHC-restricted T cell recognition is based on polymorphic peptide-presenting MHC molecules and seems to have developed for cellular immunosurveillance of cell alterations caused by intracellular pathogens or by transformation (67). The development of MHC-restricted T cell recognition that separates effector mechanisms targeted against intracellular parasites from serum antibodies recognizing soluble or particular antigens in blood and on mucosal surfaces had two major consequences: First, development of many alleles of MHC class I and class II molecules distributed in the population (MHC-polymorphism) caused histoincompatibility and immunological rejection of histoincompatible cells or organs between individuals. This histoincompatibility is of no direct evolutionary relevance, except for the immunological reactivity between mother and fetus (and in modern medicine for transplantation of organs). Because of the histoincompatibility between

maternal and paternal MHC molecules expressed by the offspring, cellular immunological memory could not be transferred from mother to offspring because of the danger of lethal graft-vs-host disease in the offspring. From this point of view and in contrast to soluble antibody memory, T cell memory is not important for the young offspring.

Second, because MHC genes and genes coding for T cell receptors are not linked (67), histoincompatibility and MHC-centered T cell recognition required a complicated developmental "learning" of T cell recognition via positive and negative selection processes in the thymus and peripherally (67). Therefore, MHC restriction of T cell recognition caused periods of immunoincompetence during ontogeny that had to be covered by transferable antibodies. Thus, antibodies are soluble, transmissible forms of a polyvalent immunological maternal memory that provides necessary protection of offspring for some duration during which they can develop T cell competence and the capacity to themselves generate essentially T help–dependent antibody responses. The requirement for an overall balanced virus-host relationship would have rendered unlikely the evolution of cytopathic agents that could not be checked efficiently by maternal antibodies during this critical time, which is needed for the full development of immune responses during gestation and particularly after birth.

A few examples may illustrate this requirement: As is known to immunologists and to those using tissue culture media containing newborn calf serum, bovine species possess no serum immunoglobulin at birth. Their B cells have not matured yet, and maternal immunoglobulins cannot pass through the complete double layer of a chorioepithelial placenta (65). The highly concentrated immunoglobulin in the first milk (cholostrum) must be taken up via gut within the first 24 hours after birth. During this period the maternal antibodies of most classes available in cholostrum are not digested and pass through the gut epithelium without degradation. This passively absorbed immunoglobulin effectively protects the calf for the first several months from otherwise lethal infections (65). Similarly, birds receive an immunological memory package of maternal antibodies in the egg (67). Offspring of mice and humans obtain maternal antibodies through the choriohemangial placenta and partially also with milk (68).

In conclusion, elevated antibody levels are essential to protect offspring via maternal antibodies not only against cytopathic infections during and just after pregnancy but more generally against the usual spectrum of all common and important infections, these are encountered not only during pregnancy but during at least the first 12 to 20 years for human mothers and 8 to 12 weeks for mice. Such infections would otherwise threaten the young host during fetal life in utero and during early infancy. In contrast to antibodies and B cells (and

possibly T helper cells) that are essential for protection of offspring against cytopathic viruses, CTLs or IL-T are not involved in antibody production and do not usually regulate antibody responses. They also do not release a product that specifically can transmit protection against cytopathic infections to offspring. Thus, they do not play an essential role in protecting offspring. Because noncytopathic viruses induce a healthy carrier state in immunoincompetent offspring, they do not jeopardize host survival. In fact survival of an immunocompetent newborn is favored by the absence in immunoincompetent hosts of T cell responses that cause immunopathological disease. Thus, the failure of adoptive transfer of CTL from mother to offspring is of survival advantage here.

METHODS TO ASSESS MEMORY

As stated, the definition of memory is variable (see introduction). If immunological memory is defined as an antigen-induced alteration in the reactive state of the immune system, then one can assess memory responses by the more rapid onset of an immune response and the capacity to reduce or eliminate the antigenic load more effectively. Therefore, clear quantifiable evidence of enhanced responses in vivo following a first encounter with antigen is needed to assess memory. If memory reflects the extent of previous lymphocyte proliferation (clonal burst size) that is maintained antigen-independently, the optimal way to measure memory for T cells is to determine T cell precursor frequencies by limiting dilution analysis (1, 3, 4). If memory represents a change in the physiological status of IL-T or CTLp, rather than simply an increase of numbers, then certain surface markers and differences in interleukin patterns should be studied (3, 11, 69, 70). If protection against secondary in vivo challenge is taken as the most important measure of memory, then the kinetics of challenging infections and of disease must be monitored in primed hosts or in adoptive transfer experiments (12, 63, 71–73). Because of the complexities of the latter parameters, these in vivo readouts are sometimes difficult to interpret. It is particularly difficult to evaluate the relevance of B cell and T helper cell memory, compared to cytotoxic T cell memory and the role of antigen persistence, under conditions where their respective importance in protection from disease or death is usually unknown or not yet clear. Nevertheless, when conditions are chosen such that a particular missing effector mechanism, e.g. memory $CD8^+$ T cells, limits survival, analysis of memory due to this particular immunological effector pathway should become meaningful (Tables 1 and 2).

In Table 1, an attempt is made to summarize and categorize immunological memory effector mechanisms: perforin-dependent cytotoxic $CD8^+$ T cells, interleukin-releasing T cells and antibodies. These protect against cytopathic or noncytopathic viruses that infect the host either peripherally (via skin, mucosa,

the big toe, or the brain) or systemically (via the blood). Evaluation of essential parameters of memory obviously depends crucially upon the particular infection analyzed. For example, protection against a particular influenza virus serotype is efficiently mediated by neutralizing antibodies. What then is the significance of in vitro measured memory at the T cell level if it does not interfere with the replication of subsequently infecting, serologically distinct influenza viruses (23, 25–27, 74, 75)? Another example may be Herpes viruses that persist in the host in so-called privileged sites, e.g. neurons, and periodically flair up to cause disease despite the presence of high titered neutralizing antibodies and a high frequency of memory T helper and cytotoxic T cells. In this example, antigen obviously persists and maintains immunological B and T cell memory at high levels, but without much success, for geographic reasons; neither antibodies nor CTLs reach and stop virus sufficiently quickly before disease becomes apparent. These may be extreme examples, but they illustrate the vast ranges of equilibrated states to which viruses and vertebrate immune systems have adjusted (15, 28, 76).

Even for classical experimental antigens used in basic immunology, the question of whether antigen maintains memory may not be as simple as it appears, because of the use of adjuvants that help via various mechanisms to render antigen more persistent (67, 77, 78). Induction of an antibody or proliferative T helper cell response with bovine serum albumin or key limpet hymocyanin is usually difficult unless these antigens are mixed with an adjuvant. For example, Freund's and now "Titer Max's" version, induce a granulomatous inflammation and cause antigen to persist in a depot (79). Alternatively, aggregates or particulate adjuvants such as alum that absorb antigens are used, so that antigen is taken up more readily by antigen presenting cells, thereby inducing an antibody response more efficiently and quickly. Thus, the antigen is probably still available—and remains available—for the formation of antigen-antibody complexes to be stored on follicular dendritic cells. In this way memory B cell responses are boosted, and elevated antibody levels are maintained.

In conclusion, whether memory is recognized and can be measured, and whether the measured parameter is essential for protection and survival, depend upon the virus analyzed and the experimental setup used. Thus, when mechanisms of immunological memory are to be evaluated, it seems essential to use readouts that monitor the effector mechanism that crucially limits reinfection and survival.

Elevated Antibody Levels

Increased antibody levels that immediately neutralize cytopathic or noncytopathic viruses are the most important memory effector mechanisms. This has been discussed in "Why Immunological Memory?" and is best illustrated by

adoptive transfer experiments, including the adoptive transfer from mother to offspring (Table 1). Elevated levels of protective antibodies depend upon antigen, provided either by repetitive subclinical infections, by viral persistence, by cross-reactive antigens, and/or by antigen-antibody complexes on follicular dendritic cells (FDC) in probably long-lived germinal centers. The evidence from various studies and infections has been summarized in comprehensive reviews by Nossal & Ada (39) and by Tew et al (13, 80). As shown in Table 1, neutralizing antibodies are efficiently protective not only against cytopathic but also against noncytopathic viruses, whenever the latter are accessible to antibodies, i.e. if the infection is via blood or mucosae or spreads systemically via blood. Antibodies are of little use against noncytopathic viruses that use routes of infection via peripheral organs such as epithelia or nerves and choriomeninges, or if they are transmitted via infected cells (e.g. HIV).

Memory T Cells Protecting Via Interleukins

Cytopathic viruses exhibit rapid replication times of four to six hours and often exhibit highly selective tropisms for only one particular host cell type (15, 16, 28). A one-cell-to-one-cell lytic CTL mechanism seems too slow to control cytopathic viruses. Soluble mediators seem necessary to block intracellular replication in extended areas where a sufficient concentration of interfering IL prevails (43, 44, 60) (Tables 1 and 2). Accumulation of interfering substances may require some time, but it does occur once sufficiently high local concentrations are established. Although this IL-mediated pathway may be imprecise, it seems to harm mostly (only?) cells already hampered in their function by infection. Compared to cytolytic T cell action, this effector mechanism may therefore overall be considerably less dangerous to the host (see next section).

One prototype example of the effect of protective IL–T cells is the protection they provide against peripheral reinfection with vaccinia virus via mucosa or skin (see von Piquet's experiment). However, since this experiment shows that neither neutralizing antibody nor presumably primed $CD8^+$ or $CD4^+$ T cells prevent virus from replicating locally 4 weeks after priming, it emphasizes that only antibodies are critical for preventing systemic spread of virus. Memory IL–T cell responses beyond that of a primary response are therefore not essential for survival. Nevertheless, an efficient nonlytic but IL-dependent protective T cell function against vaccinia virus is mediated by activated $CD8^+$ T cells and also by activated and therefore tissue-seeking $CD4^+$ T cells (44). Interestingly, elevated memory frequencies of nonactivated T cells are apparently inadequate to mediate efficient protection against such local peripheral challenge infections even when the frequency is 10^2 to 10^3 times greater than normal. If T cell frequencies are unphysiologically high such as in TCR transgenic mice (81, 82), a delayed activation may still result in some protection because of the enormous

numbers of quickly inducible IL-T cells, but in general not even such mice are protected against peripheral infection. A second example is rhabdovirus infections [i.e. rabies or vesicular stomatitis virus (VSV) infections] or infections by influenza and some parainfluenza viruses (e.g. Sendai virus infections in mice). Local neutralizing antibodies seem to be the critical protective mechanism preventing reinfection, apparently independently of increased cytolytic CTLp frequencies as determined in vitro (23, 83, 84). Thus, although increased CTLp and IL-T cell precursors can be measured, and protective activity can be demonstrated under selected conditions in vivo, they do not contribute to the long-term immunological memory phenotype that is critical in mediating resistance against disease and that is important for survival (85).

Evidence from VSV and influenza model infections in mice may illustrate this point particularly well—in a negative fashion—for primed T helper cells. Neutralizing antibodies are critical to limit infection and to protect against reinfection. T helper cells are necessary for switching neutralizing antiviral IgM to IgG, when induced by various influenza viruses or by VSV New Jersey (NJ). T helper cells extensively cross-react among influenza viruses (30, 86–88) and between VSV-NJ and Indiana (IND) (89–91). Surprisingly, despite this T cell cross-reactivity, no significant cross-protection is noticed. The time-dependence of cross-reactive memory T helper cell activity was assessed after infection with VSV-NJ by monitoring the acceleration of the switch from IgM to IgG in the same mice when they were subsequently exposed to VSV-IND. Functional memory T help that could accelerate this switch, specifically beyond that of a primary response, lasted only 14–21 days (92). Interestingly, this was despite the fact that T helper cell responses assessed with a classical hapten carrier system using the same viral carrier lasted for months (89). Thus, functional T cell memory seems to be rather short-lived against VSV and, similarly, against influenza virus (30), despite the presence of elevated numbers and frequencies of primed T helper cells. This suggests that, despite persistence of antigen-antibody complexes on FDCs, T helper cell memory drops rapidly below a minimal level, at which the induction of available naive B cells specific for the new serotype, rather than T helper cells, is limiting. Obviously, these cross-reactive T cells are not controlling the infection, and this fits with what we know, that only neutralizing antibodies are efficient in doing so. The very fact that serotypes of these viruses did evolve is good proof for the relevance of long-term memory B cells and antibodies in these particular infections. This study of functional half-lives of cross-reactive T helper cell memory shared by different viral serotypes reveals a particularly interesting example of the coevolution of immune responses and viruses, and it illustrates how important a biological readout is to measure critical parameters to learn about immunological memory.

Cytotoxic T Cell Memory

PROTECTIVE ROLE OF PERFORIN-DEPENDENT CYTOTOXIC T CELLS Specific T cell–mediated and perforin-dependent host cell lysis is the critical effector mechanism against cells infected with noncytopathic viruses or against transformed cells. Noncytopathic viruses replicate rather slowly, with average replication cycles of about 10–20 hours (28, 50). They are relatively resistant to IL-dependent effector mechanisms and often exhibit a very wide spectrum of tropisms that permit them to infect many different host cells (49–51). CTLs seem to be essential for control of these viruses, a point revealed by recent studies with perforin-deficient mice; such mice are unable to stop or eliminate LCMV (54). Since these viruses are noncytopathic, they cause disease or death not directly, but indirectly via the protective CTL response (see Introduction and Table 1); therefore limitation of CTL- and perforin-mediated immunopathology is essential for protection and survival. It is noteworthy that CTLs often do not succeed in eliminating noncytopathic viruses completely from the host. Even when no longer measurably detected in lymphoid or many other organs, LCMV has been found often to persist in kidney tubular cells, in testes, or in salivary gland cells (49–51, 61). From these cells virus may spread periodically to boost CTL responses in lymphoid organs without inducing an effector CTL population potent and numerous enough to reach the peripheral sources of virus. These notions are still poorly defined by quantitation but may be illustrated in the following experiments, with transgenic mice expressing the LCMV-glycoprotein (LCMV-GP) under the rat insulin promoter in insulin-producing islet cells. T cells of these mice ignore this transgenic self-antigen and do not spontaneously develop diabetes (94). However, after infection with LCMV, the mice develop a prompt LCMV-GP specific CTL response, which causes the destruction of the peripheral transgenic islet cells and initiates a fulminant diabetes. In contrast, infection of the same mice with a vaccinia recombinant virus expressing LCMV-GP fails to cause diabetes (95), despite the fact that it induces an LCMV-GP-specific CTL response (about 100 times reduced compared with that induced by an LCMV infection). LCMV persistence in kidney tubular cells may be comparable to LCMV-GP transgenic islet cells exposed to a low frequency CTL response; it is not reached efficiently by the few activated memory CTL.

Thus, noncytopathic viruses tend to persist in a host because they are relatively resistant to IL-dependent mechanisms. Because of their multiple cell tropism, they can persist in immunologically privileged sites to coexist in a balanced fashion with few activated memory CTL (and antibodies).

CYTOTOXIC T CELL PRECURSOR FREQUENCIES DETERMINED IN VITRO CTL memory is often monitored by secondary cultures in vitro (1, 3, 83). Usually unprimed spleen cells cannot be stimulated with antigen-expressing stimulator cells to generate measurable CTL activity in vitro. Although this statement still applies in general, in the past several years, reports have emerged on induction of primary CTL responses in vitro, when appropriate sources of interleukins, the best APCs, and sufficient numbers of normal responder lymphocytes are used (96, 97). Therefore, the presence or absence of a secondary response may no longer distinguish absolutely between primed and virgin CTL. Nevertheless, in every case where CTL memory has been studied early after antigen exposure, increased CTLp frequencies have been observed.

After infection with various viruses, the CTLp frequency increases dramatically from 10^{-6} spleen cells to peak values of 10^{-2} or 10^{-3}. As soon as a virus is eliminated to levels below detection, CTLp also usually drop to 10^{-4} or 10^{-5} within a few days, but they usually do not drop back to 10^{-6} (1, 3, 63, 83, 98). These findings signal that antigen-dependent homeostasis prevents accumulation of irrelevant or no-longer-used effector $CD8^+$ T cells over time. There are a few examples where CTLs seem to persist at rather high levels in unmanipulated hosts or after adoptive transfer of CTLs to irradiated recipients, in the apparent absence of antigen, for various reasons to be discussed later.

What regulates this long-term CTLp increase? Is it genuine antigen, that persists from the first infection or derives from reinfections, cross-reactive antigens, or bystander activation processes, or does it represent a true memory quality that is built into the immune system?

$CD8^+$ T CELL–DEPENDENT PROTECTION ASSESSED IN VIVO Since in vitro measurable elevated CTLp frequencies are found after most virus infections, independent of whether these CTLs are essential for protection or not, CTL protection in vivo and the role of antigen in maintaining protective CTLp memory need to be analyzed carefully in vivo to find conditions where memory CTLs acting via perforin-dependent mechanisms are critical for protection.

CTL memory has been studied in various model situations in the original host or by adoptive transfer experiments to document longevity or, alternatively, the transient nature of immunological memory and its dependence upon CTLp frequency, either alone or also upon activation with persisting antigen (1, 3, 63, 72, 73) (Table 2). Adoptive transfer experiments of antibodies documenting the critical role of neutralizing antibodies in protection against reinfection are easily done because no additional treatment of the host is necessary. In contrast, if CTL memory is analyzed in the original host, quantification is difficult; remaining antigen associated with transferred cells may not be eliminated, antibodies may interfere, etc. Nevertheless the original primed host and the prevention of

immunopathology are critical subjects of research on the nature and relevance of CTL memory (48, 63).

If persisting antigen is removed by the isolation of lymphocytes from immunized donors and then transfused to recipients that had been pretreated either by sublethal irradiation or by other methods causing immunosuppression, several methodological problems arise. How do lymphocytes taken out of their physiological context circulate and home in such a new recipient (99–103)? Do they expand, and to what degree? How do activation markers, surface molecules of donor cells, and homing receptors on donor and recipient cells vary because of irradiation and adoptive transfer (102)? Is a recipient inbred mouse really genetically identical to the individual donor mouse to the degree usually assumed, or are these assumptions incorrect because of highly variable endogenous or integrated viruses (104–109)?

Already in early adoptive transfer experiments on memory, Celada et al (12) noted that transfer was difficult or impossible unless recipients were first irradiated with 450 and 650 r, i.e. conditions that cause a rather dramatic depletion of lymphocytes with subsequent expansion of endogenous or transferred lymphocytes. Therefore, this experimental protocol provides conditions of activation of transferred memory cells to an extent that is not controllable (1, 3). Whether irradiation in addition causes activation and expression of endogenous viruses with potential superantigen character may be too speculative at the moment, but this disturbing possibility may have to be considered. In this respect, inbred strains of mice may be particularly problematic because they harbor a rather extensive variety of such endogenous or slow viruses, for example, the radiation-induced and other leukemia viruses, MMTV viruses in various shades and transmission forms, or endogenous viruses involved in encephalitis (106, 108–112).

As stated before antibodies play a major role in preventing systemic reinfection and disease with cytopathic (or noncytopathic) viruses. Immunological memory mediated by IL-T has been discussed in relation to the vaccinia virus model infection in previous sections; for the discussion of CTL memory the noncytopathic LCMV is used as a well established animal model infection. LCMV does not cause disease directly, but via CTL-mediated immunopathology, in spleen, or liver, or in choriomeningitis. CTL memory is essential and limiting for protection against peripheral infection with noncytopathic viruses. What are the parameters that influence protection by memory $CD8^+$ T cells against virus-induced immunopathology? CTLp frequency or antigen-dependent activation of CTLs, or both? Protection by CTL has been tested in vivo usually in two ways: First, by determining if preexistent or transferred primed CTL reduce viral titers after a challenge infection more efficiently than do unprimed CTL. However, since perforin-mediated T cell cytotoxicity is not critical for

protection against cytopathic viruses, this test does not yield information relevant from an evolutionary point of view about memory against cytopathic viruses. Second, cytolytic T cells cause disease via induction of T cell–mediated immunopathology against otherwise benign (noncytopathic) viruses. Therefore, protective memory against disease caused by noncytopathic viruses is most appropriately monitored by assessing prevention of immunopathology. This parameter determines the survival of the host. For example, intracerebral injection of the noncytopathic LCMV causes viral spread in the choriomeninges, and subcutaneous infection causes spread in the footpad. These cells are susceptible to $CD8^+$ T cell–mediated immunopathology (48–51, 113, 114). Only activated cytotoxic $CD8^+$ T cells protect against lethal choriomeningitis or sizable footpad swelling (63, 115–117). While increased CTLp frequency in the absence of persisting antigen protects inadequately in this assay, it still exhibits measurable protection in a virus titer reduction assay measuring systemic virus levels in the spleen (3, 63, 117, 118). Since LCMV is noncytopathic, disease is not directly caused by the virus but is mediated by T cells. Therefore, virus titer reduction may be important, but the parameter limiting disease and death is in this case the prevention of immunopathology.

In conclusion, from a biological point of view, protection against noncytopathic viruses is probably best measured by prevention of immunopathology. In contrast, protection against cytopathic viruses is best measured by assessing virus titer reduction. These drastically differing aspects of antiviral immunity are based on the exemplaric and extreme differences in the life styles of the viruses and of the limiting immune effector mechanisms.

SPECIAL CASES REVEALING CHARACTERISTICS OF CTL MEMORY

Very high CTL precursor frequency in TCR transgenic mice The role of memory T cells, of their frequency, and of their activation in memory against cytolytic and noncytolytic viruses was analyzed in mice expressing a LCMV-GP and $H2D^b$-restricted transgenic T cell receptor (81, 82). These T cell receptor transgenic mice exhibit extremely high frequencies of LCMV-specific $CD8^+$ T cells (about 1 of 2 of their $CD8^+$ T cells are LCMV-GP plus D^b specific). They are protected against an i.v. challenge infection with 10^2–10^4 pfu of LCMV; virus is not measurable in spleen or liver by day 2 or 4 after infection, whereas in control mice high virus titers are seen (82) (Table 2). The same mice are not always protected against intracerebral infection with this noncytopathic virus or by infection in the footpad; the variability of the results and the fact that some mice survive is probably not due to direct protection but rather reflects the fact that an intracerebral infection cannot be easily initiated without simultaneous systemic infection reaching lymphoid organs and activating CTLs

immediately. Mice die within 2–4 days or develop a significant immunopathological footpad swelling reaction around day 2–4 (116). In contrast, several experiments have shown, in LCMV primed mice and in primed TCR transgenic mice, that high frequencies of activated $CD8^+$ T cell precursors are capable of protecting mice against lethal choriomeningitis. This demonstrates that high, even extremely high, precursor frequencies of specific CTL alone are poorly protective against cytolytic T cell–mediated immunopathology. Preactivation of these lytic effector T cells that enables them to emigrate into the periphery to monitor infected cells immediately upon infection seems critical. If transgenic effector T cells are preactivated by LCMV, challenge virus is controlled so fast that the little immunopathology induced is insufficient to cause overt disease. Comparably also, if transgenic mice are challenged with a vaccinia recombinant virus expressing the relevant peptide, they are not protected at an early time against viral growth in the ovaries (119). However, later, on day 4–6 after challenge infection, they may control the challenging virus slightly more efficiently than do control mice. This result obviously contrasts with the capacity of the TCR transgenic mice to control virus titers in spleens after LCMV challenge infection. This discrepancy requires an explanation.

The difference in memory T cell requirements between i.v. and peripheral challenge infections can only be understood with the knowledge of the relative kinetics of the effector $CD8^+$ T cell reaching infected cells vis-á-vis the kinetics of the disease process (i.e. numerics and kinetics of cell infection and damage by the cytopathic virus or immunopathological destruction of host cells infected by noncytopathic virus). These kinetics are crucial to the evaluation of the biological role of $CD8^+$ T cell memory. The findings on TCR transgenic mice illustrate that nonactivated $CD8^+$ T cells (i.e. antigen-independent high frequency CTLp) can limit virus replication efficiently against noncytopathic viruses (or tumors) in spleens, where they are activated by antigen and can immediately mediate effector function. (As pointed out earlier, systemic replication is also controlled very efficiently by neutralizing antibodies—120, 121). However, to avoid lethal immunopathology by cytolytic T cells after peripheral infection, $CD8^+$ T cells have to be preactivated and able to immigrate into tissues very fast; since activation is antigen dependent, protective CTL memory is also antigen dependent. Thus, unless lytic CTL are preactivated, the delay between virus spread from peripheral tissue sites to organized lymphoid tissue (where high frequency resting memory T cells are then activated) is sufficient to permit virus to spread so far that subsequent immunopathological damage is extensive enough to cause disease.

These same experiments also demonstrate that while intravenous infection of mice with the slowly replicating LCMV permits relatively rapid activation

of T cells and therefore reduction of virus titers by day 2 in spleens, this is apparently not the case against the more rapidly replicating cytopathic vaccinia recombinant virus. In this latter case, $CD8^+$ T cell memory seems not really efficient and important to protect against cytopathic viruses (a point discussed here in the section on Memory T Cells Protecting Via Interleukins, and in the paper by Ramshaw and collaborators—43). This conclusion parallels the recent demonstration for vaccinia virus that perforin-dependent target cell killing seems to be of little relevance to control cytopathic viruses, whereas interleukin-dependent protection is key (60). Interleukin-dependent T cell memory can catch up with virus spread despite delayed kinetics because effector function is not mediated via individual cell contact, but rather by diffusable and exponentially increasing protective factors such as interferons, tumor necrosis factor, etc.

Therefore, dependent upon the kinetics of virus replication, sites of infection, and whether virus causes direct cytopathogenicity or triggers immunopathology, T cell memory is activation- and therefore antigen-dependent in the cases of peripheral infection. It is not necessarily activation and antigen-dependent when reinfection dominates initially in lymphoid tissue, where resting memory T cells are activated efficiently and directly at the site of needed action. However, as stated earlier, since neutralizing antibodies can efficiently protect against systemic infection caused by cytopathic viruses, neither interleukin-dependent nor perforin-mediated antiviral protection by memory T cells, beyond that of a primary response, is usually essential for survival after i.v. challenge infections.

Neutralizing antibodies in absence of memory CTLs Some interesting exceptions occur when the presence of neutralizing antibodies alone may modulate virus-spread in a detrimental fashion, and under special conditions where cytotoxic T cell–mediated immunopathology is a key pathogenetic principle. These exceptions may further illustrate the concepts developed in this review. However, it must be emphasized that these conditions do not usually occur under normal field conditions they may, however, become apparent under special vaccination conditions where neutralizing antibodies are induced in the absence of CTL. Respiratory syncytial virus infection in humans (16, 122), feline leukemia virus in cats (123–125), and LCMV in mice may become apparent in the presence of neutralizing antibodies; but in the absence of cytotoxic T cells, such viruses may cause a more serious disease than occurs in control individuals (126). Under these conditions, induction of protective CTL is inhibited in lymphoid tissue by the presence of neutralizing antibodies, while virus nevertheless spreads peripherally in critical cells in solid tissue (126) (Table 1). When the delayed T cell response arises, the ensuing T cell–mediated pathology may be considerably more extensive than in a host without neutralizing antibodies (see also Table 1).

A small increase of lytic CTLp by vaccination may enhance immunopathology Immunization of mice with vaccinia recombinant viruses expressing either nucleoprotein or glycoprotein of LCMV, or alternatively, with relevant T cell peptides, protected these mice against low-dose infection of the choriomeninges. This vaccination rendered them, however, more susceptible to infection with more rapidly replicating LCMV-isolates. In nonimmunized mice, these isolates failed to cause a lethal CTL-mediated immunopathology because of a process called exhaustive induction and depletion of CTL (see the section on Exhaustive Induction of $CD8^+$ T Cells and Memory).

Immunization with the two vaccination protocols reduces the virus load and relative distribution in lymphoid tissue versus in the periphery. The T cell response is sufficient to control slowly replicating LCMV-isolates efficiently, thus preventing CTL-immunopathology before the lethal disease develops. In contrast, fast-replicating LCMV-isolates that otherwise could have outrun the CTL response and would have exhausted it are now partially controlled because of weak vaccination. They thus are slowed down in their spread, rendering them incapable of exhausting the CTL response. Since these viruses spread in the periphery, a potent CTL response now can cause lethal immunopathology (62, 64, 127).

Under these selected circumstances, weak or partial vaccination is harmful to the host. This example demonstrates again that the relative kinetics of virus spread in the periphery and of the memory CTL response are critical for protection against immunopathology and for survival.

Taken together the three special examples show that the question of what parameters (frequency, activation, or both) influence protection by memory lytic CTL against virus-induced disease may be answered as follows. Protection against reinfection by noncytopathic virus in the periphery in solid tissue is not mediated by antibodies. Rapid migration of CTL to peripheral tissue is essential, and therefore preactivation of memory CTLs is necessary for protection against immunopathology. Even after low-dose infection, noncytopathic viruses (e.g. LCMV, HBV) may persist in the host, particularly in immunologically privileged sites such as kidney tubular cells, testes, and salivary glands, from where virus can spread periodically to lymphoid tissue (probably also in the presence of, or even enhanced by, neutralizing antibodies). This persistence is necessary to keep memory CTLp activated, and these can keep persistent viruses under control. Against cytopathic viruses, antibodies are necessary and usually sufficient. Protection may also be documented for memory T cells if activation by challenging antigen and protective interleukin accumulation occurs. However, from an evolutionary point of view, these latter effector memory pathways are probably not usually essential for cytopathic viruses.

Elevated CTLp frequencies and an increased activation status cannot usually be separated completely. In particular, an increased activation status of CTLs always goes along with increased CTLp frequencies (TCR transgenic mice therefore are an exception). In addition, and as outlined previously, even very high frequencies of CTLp do not efficiently protect against peripheral viral infections in the absence of preactivation, in particular for cytopathic viruses such as vaccinia viruses, because of rapid virus replication and the particular kinetics needed to build up necessary concentrations for interleukin-mediated protection. These examples emphasize again that one should test immunological memory only under limiting conditions in a readout that is essential for the coevolutionarily defined virus-host relationship. If one accepts such a viewpoint, immune memory specific for viruses such as LCMV (and probably HBV, HCV, HIV) must be studied under conditions preventing overt $CD8^+$ T cell–mediated immunopathology. Against cytopathic viruses that are not readily controlled by local antibody levels such as pox virus infection in the skin, one probably best studies local virus titer reduction; however, the overwhelming importance of memory B cell and antibody levels for survival of the species cannot be overemphasized.

A Comparison of In Vitro Measurable Cytolytic $CD8^+$ T Cell Memory with Immunity In Vivo?

In vitro restimulation of $CD8^+$ memory T cells to assess either CTLp frequency or primed T cells often differs from in vivo tests fundamentally with respect to time intervals involved. Whereas in vitro secondary cultures last for 5–7 days, protection assays assessing antiviral protection in vivo use 1–8-day intervals. Obviously, the various assays are not measuring the same thing. A comparison has been summarized recently and therefore needs only brief reference here (83, 128). Mice primed with increasing numbers of LCMV-GP–expressing stimulator cells revealed the following hierarchy of qualitative differences according to various tests to assess $CD8^+$ T cell memory in the original host. An in vitro restimulation of CTLp, using ^{51}Cr released from infected target cells as readout to measure cytotoxicity, was the most sensitive test because it required the smallest vaccine dose. More priming antigen was required for LCMV titer reduction in spleens of primed mice after i.v. challenge infection. Induction of $CD8^+$ T cells that efficiently protect recipient mice against a challenge infection with recombinant vaccinia expressing LCMV-GP in a solid peripheral organ such as the ovary was even more demanding. The most demanding was induction of memory $CD8^+$ T cells capable of protecting recipient mice against choriomeningitis or swelling of footpads after local infection (83).

In conclusion, the relative importance of lytic CTLp frequency alone or in combination with activation in immunological memory depends upon the model system used. Interestingly, except in prevention of CTL-mediated immunopathology, the models measuring CTL memory probably do not adequately represent conditions that are relevant for protection against reexposure to cytopathic viruses such as measles, pox, or polio viruses. Here challenge doses usually are low, and neutralizing antibodies prevent disease efficiently. Thus, one may question the relevance of long-term $CD8^+$ T cell memory in these infections. During the phase of high risk of reexposure to the same infection, i.e. during epidemics, antigen from the waning infection usually guarantees high levels of CTLp plus activation by antigen and thereby provides efficient protection by CTL in addition to that provided by neutralizing antibodies.

The Role of Activation in CTL Memory

Whenever T cell memory has been analyzed, it has been correlated with a special memory quality reflected by various surface cell markers. Detailed reviews of those parameters have been presented and need not be repeated (11, 69, 101, 102, 129–132). The disappointing result of most studies is that although several markers separate naive (unprimed) T cells from primed ones, no real memory markers reliably distinguish activated from nonactivated memory T cells. Thus, no obvious marker permits a distinction between effector T cells or activated primed T cells and memory T cells; it is possible that no such distinction can be made. The important role of adhesion molecules on effector T cells and memory T cells for migration into peripheral tissues has been summarized extensively by Butcher (133) and MacKay (8, 9); an earlier review by McGregor & Kostiala (134) stresses this aspect clearly. The evidence suggests that activation is necessary for T cells to express some, and to downregulate other, molecules to be able to emigrate into solid tissues. Naive T cells or nonactivated T cells cannot do this; but rather they recirculate from blood to lymph nodes via high endothelial venules (HEV), avoiding peripheral tissues (135) (Table 3).

Thus, migration of memory T cells through tissues to act against peripheral infectious foci depends upon their being activated. If the infectious agent reaches lymphoid tissues directly, and at relatively high concentrations, then nonactivated CTLp are activated within short periods of times to emigrate through tissue and mediate effector functions peripherally. Although some infectious agents reach blood and lymphoid tissues early, this is not a general rule (15, 136). Most infections start peripherally, and then the infectious agent reaches first to local lymphoid organs where priming starts, and then spread further via lymph or blood. An example for this is the following model situation in mice expressing the transgenic T cell receptor specific for the LCMV-GP plus $H\text{-}2D^b$ on more than 90% of T cells. When the specific peptide was injected

Table 3 Characteristics of B and T cell memory

	Memory B cells		Memory T cells	
	Resting	Activated	Resting	Activated
Location/Migration	recirculation blood→LN (via HEV)	associated with persting antigen in lymphoid organs and bone arrow	spleen and blood possibly migration blood→LN (Via HEV)	migration through tissue
Function	secondary reaction upon challenge	maintance of memory IgG level	secondary reaction upon challenge	immediate killing of infected cells in periphery
Timeframe of response	delayed	immediate	delayed	immediate
Proliferation	no	yes	no	probably yes
Site of proliferation		GC		probably within lymphoid organs associated with ag
		GC		
Antigendependence	no (poor)	yes	no	yes
Site of antigen persistence		FDC		not known

into footpads of such primed mice, local footpad swelling as a reflection of specific CTL activity was measurable by 10 to 18 h in primed mice, but in unprimed mice only after 20 h, peaking by 28 h (116). This supports the view that in unprimed mice, peptide first induced effector T cells in local lymph nodes, and effector T cells then migrated to peripheral tissue. Therefore, this example illustrates again that, as a rule, increased CTLp frequency alone cannot immediately and alone mediate memory functions against peripheral infections; either preactivation or new activation seems necessary. The key questions asked in nontransgenic situations are: How is the frequency maintained at high levels after priming (compared to in nonprimed mice)? And, is increased CTLp frequency alone capable of providing protection, under which conditions, and against what type of virus?

A recent study with the H-Y–specific TCR transgenic mice revealed in adoptive transfer experiments and by monitoring activation markers that memory cells could be derived from activated T cells and persist in the absence of antigen in adoptive recipients for at least 13 weeks (69, 137).

It is appropriate to conclude here that frequencies are important, but that activation seems necessary for memory CTLs to mediate evolutionarily important effector functions limiting viral spread in the periphery and host cell damage by immunopathology.

EXHAUSTIVE INDUCTION OF CD8$^+$ T CELLS AND MEMORY

When antigen is meeting the immune system in a localized fashion (e.g. via locally initiated infection), T cells are induced in local organized lymphoid tissue (138, 139). If antigen does not reach organized lymphoid organs—usually via APC—the antigen is ignored by T cells and no response is induced (94). If antigen is quickly and very widely distributed through the immune system and the host, antigen may induce all precursor T cells within a few days. Because induced effector T cells (and plasma cells) die usually within a few days after induction, the result of this overwhelming induction is that this particular specificity is deleted and becomes undetectable functionally in the host (140). Similar results can be obtained with superantigens (141, 142). Exhaustion of CTL is probably a very important normal process that guarantees lymphocyte homeostasis after induction of specific T (and B) cells; partial exhaustion may be seen during normal immune responses in an incomplete way. As stated before, after viral infection CTL are induced, and expand from a frequency of 10^{-6} in spleen to maximal CTLp of 10^{-2} to 10^{-3}. By the time measurable virus has disappeared, CTL frequencies drop to 10^{-4}–10^{-5}. Thus $>$ 99% of

induced T cells seem to die in any immune response as soon as the antigen disappears.

The mechanisms leading to complete exhaustion of a CTL repertoire are still largely unclear. Nevertheless, phenomenologically the data indicate that either overstimulation under special conditions or stimulation of all available precursors within a few days favor exhaustion. Since exhaustion with superantigens involves a class II MHC-dependent mechanism, it seems unlikely that insufficient levels of T help are a key mechanism for enhancing exhaustion in lymphoid organs. T help may be a limiting factor for CTL exhaustion as shown after overwhelming infection of mice with LCMV, where both APC and peripheral class II^- cells are infected. One possible mechanism of exhaustion of CTLs may be starvation of IL in the periphery outside of nonlymphoid organs, but this is largely unexplored. Alternatively, Welsh and collaborators suggest that available high levels of IL in lymphoid organs may enhance lethal proliferation of induced T cells (143).

The finding of exhaustive induction of CTLp raises basic questions about the nature of CTL memory, because this finding seems to render reversion of effector T cells to memory cells rather unlikely. Are memory T cells then a consequence of unequal divisions after induction? If there is a special memory quality of CTL, how is the decision made by the immune system that an induced T cell should be only "half"-induced to become a memory T cell, and not fully induced, because then they apparently die off. Alternatively, is there a possibility that a fully induced effector T cell, apparently about to die, will not die, but instead reverts to become a memory T cell? A speculative alternative, more favorable to the notion of "half"-induction, may be that T cell induction decreases as antigen decreases, so that by the end of a controlled infection, induction slows and then stops at the time when antigen is virtually completely eliminated; interleukins may also decline and freeze a few T cells just on their way to be induced in an intermediate memory situation. Conceptually the simplest possibility seems to us that initial antigen-kinetics and amounts and kinetics of antigen-elimination together regulate the frequency, while the levels of persisting antigen regulate the activation of memory T cells.

HOW DOES ANTIGEN PERSIST?

Persistence of antigen on FDC is well documented, and such persistence is thought to maintain B cell memory, leading to long-term elevated antibody levels in serum (40). Whether the same antigen also maintains T helper memory is possible, but less clear. Cytopathic infectious agents are usually rapidly eliminated completely (e.g. pox), but reexposure or persistence as incomplete viral information may boost and maintain the response. Other viruses may persist

in a latent stage in selected cells, where they are temporarily not cytopathic (e.g. Herpes viruses). On the other hand, noncytopathic agents may persist in peripheral sites, where they are not reached easily even by a few activated T cells (95).

Usually remaining persisting viral foci are effectively controlled in an ongoing immune response. This important mechanism was recognized for mycobacterial infections and LCMV in the 1960s and was termed "infectious immunity" (144, 145). This is well illustrated by the finding that T cell memory in humans against a few persisting infectious agents can be demonstrated by delayed hypersensitivity: Injection of tuberculin elicits a swelling in vaccinated or infected patients. Similar skin tests were evaluated for several other pathogens but found only for those inducing granulomas, such as tuberculosis, brucellosis, leprosy, and histoplasmosis, where pathogens and their components induce granulomas and establish antigen-depots that remain in the host for years, maintaining activated T cell responses. Similarly, protein-antigens induce long-lived DTH only if given in complete Freund's adjuvant, which also forms granulomas (77, 79, 146).

There is some evidence that a similar mechanism may be involved in acute LCMV-infected mice and possibly in HBV-infected patients where virus obviously persists (28). HIV is a prominent example of infectious immunity involving CTL (besides neutralizing antibodies), essentially controlling the balance between virus and the host (28, 147, 148). These findings suggest that persistence of weak or noncytopathic infectious agents usually is accompanied by a low level of activated effector T cells, which guarantee an acceptable balance with the virus, thereby keeping immunopathology at minimal or tolerable levels. Once this balance gets disturbed or out of control, chronic, subacute, or acute immunopathology may become overwhelming and may cause lethal immunopathological disease; examples are forms of aggressive hepatitis and probably HIV-induced AIDS in their various disease kinetics and phenotypes (53).

Antigen may persist in other forms, for example as antibody antigen complex on follicular dendritic cells. We have previously shown that VSV-protein-antibody complexes persist for several weeks in draining lymph nodes (103, 149) and that immunization with soluble VSV-NP induced CTL responses (150). Combined, these may suggest that follicular dendritic cells drive not only B cell memory but possibly also CTL-mediated immunity. In this light, the fact that exogenous protein antigens enter the class I pathway only inefficiently may be a mechanism to conserve this minimal antigen-depot (40). Protein-antigens derived from virus infections appear generally to persist in draining lymph nodes for weeks to months and could serve this postulated purpose. However,

recent experiments using IgM knockout mice render this mechanism unlikely because these mice seem to exhibit a normal CTL memory. Alternatively, antiviral immunity could be maintained by class I MHC-bound peptides persisting per se. Peptides with surprisingly long half-lives (up to 600 h) have been identified, and MHC molecules on dendritic cells may be very stable (151–153).

Nonspecific Restimulation of Memory CD8$^+$ T Cells by Cross-Reacting, Mimicking Antigens, or by Bystander Effects

That specific antigens are not necessary to maintain CTL memory has been explained as reflecting restimulation by cross-reaction or the mimicking of antigen (2, 3, 154). Accordingly, memory CTLp need antigen–MHC interactions that are less avid than required for priming and therefore related peptides that are similar or that mimick the unique specific antigen (or super antigen) may keep CTLp activated. This seems to be a rather theoretical argument that lacks direct evidence (1–7).

Alternatively, memory CTL may be boosted periodically by bystander effects, essentially mediated by interleukins, particularly IL-2. Accordingly, interleukins released during activation of T cells specific for antigen may maintain proliferation/activation of memory T cells that previously had been activated by antigen. Again, evidence that such mechanisms play a major role in maintenance of memory is incompletely explored at the moment. Nevertheless, the question whether conditions that enhance expansion of lymphocytes, potentially including also CTLp, is however relevant to the discussion of memory. As discussed in the previous section, the finding that transfer was difficult or impossible unless recipients were first irradiated with 450 and 650 r may suggest that this experimental protocol provides conditions of activation of transferred memory cells to an extent that is not controllable (1, 3, 11), and therefore it may yield inadequate information when the role of precursor frequency and of activation needs to be analyzed.

STRIKING PARALLELS BETWEEN B CELL/ELEVATED ANTIBODY MEMORY LEVELS AND T CELL "MEMORY" (TABLE 3)

The evidence summarized reveals striking similarities between T and B cell memory, the two complementing major specific arms of the immune system.

Activated memory T cells are, as discussed, necessary to control replication of cytopathic virus in the periphery and for the prevention of immunopathology triggered by noncytopathic viruses. Since memory T cells cannot be

transmittted to offspring, they are of limited importance for survival of the species. In contrast, elevated memory antibody levels are critically important; they protect against virus infections and are transmitted to offspring. Thus, the overall evolutionary significance of protective antibody levels is in a different category than is protective CTL memory. It is not established whether elevated frequencies of recirculating memory B cells are antigen-dependent (103, 155). However, as stated before, maintenance of elevated memory antibody titers is antigen-dependent (40). This is illustrated by the finding that 1. Antigen persists in germinal centers on FDC for a long time and causes B cell proliferation (156), and 2. B cells transferred to a host in absence of antigens stop making antibodies (71). Thus, in general, elevated B or CTL precursors in blood or spleen are antigen-independent, but both protective memory mediated by B cells and elevated antibodies (and probably T helper cells) and protective memory by T cells are antigen-dependent. Antigen dependence of both effector mechanisms is critical for survival of the species (i.e. elevated antibodies) and of the individual (activated IL-T and CTLs), and it renders these responses easily regulatable because effector T cells and effector B cells (plasma cells) have a short half-life; accumulation of T cells or B cells that are not needed any longer is avoided. The balance between immune response, reducing antigen depots, and continuous need for induction of sufficient numbers of activated effector CTL and B cells apparently keeps memory continuously at an optimum level. Evolutionary selection for some antigens to persist prevents memory from being preoccupied with irrelevant antigens. If true "special memory" T cells existed, then the regulation would probably require rather demanding and complicated regulators, as have been postulated in the form of suppressor T cells, cellular or idiotypic networks. The proposal that antigen alone regulates immune responses, including memory, may be the simplest and most attractive concept. Although shown convincingly for B cells, and suspected but not proven for $CD4^+$ T helper cells, evidence is admittedly less direct for CTL, but as reviewed here, a convincing case can be made that noncytopathic viruses are kept in check via activated CTL.

Not surprisingly, antigen dependence of both memory B and T responses is compatible with many characteristics shared by effector and memory T and B cells, and corresponding similarities are also shared by resting B and T cells (Table 3). The quotation marks for memory are used now because of the difficulties in defining a "true memory" status. Protective memory seems to reflect low frequency antigen-dependent activated B and T cells that are not fundamentally different from acutely activated effector T and B cells. "Memory" and activated T cells emigrate into solid tissue; resting T cells do not. Activated B cells expand in germinal centers upon contact with antigen on FDC

and emigrate to bone marrow; resting B cells do not. Both resting B and T cells recirculate through blood to spleen and via HEV to lymph nodes but do not reach germinal centers or solid peripheral tissues, respectively. Both activated B cells and T cells are ready for immediate or continuous action, which is necessary to fulfill the respective requirements for protection of the species population (antibodies against destructive viruses) and of the individual host (early CTL protection to avoid destructive immunopathology).

CONCLUSION

It is obvious from previous discussions and this overview that our knowledge of the various facets of virus-host relationships is still incomplete. However, a basis exists from which to understand the different aspects of immunological memory. The various infectious agents are checked by distinct immunological effector and memory pathways that are in balance with many other parameters of the infectious agents such as host range, tropism, cytopathogenesis, replication cycle, and dormancy. Protective antibodies, because of their transmissibility from mother to offspring, dominate immunological memory. Neutralizing elevated memory antibody levels are efficient and sufficient to limit reinfection to foci in solid tissue and to prevent hematogenic spread. Therefore, nonlytic memory T cells are not essential for protection against reinfection. Neutralizing antibodies efficiently controls hematogenic spread. However, if noncytopathic virus spreads too widely in solid tissue where neutralizing antibodies have no access, then immunopathology may become life threatening to the individual. In these infections, which are also poorly controlled by interleukins, an efficient CTL response is crucial to limit potentially lethal immunopathology; to achieve this goal antigen-activated primary or "memory" CTL seem crucially necessary. In conclusion, antigen-activated memory CTL are not different from effector CTL, and since antigen-activated B cells/plasma cells are needed to maintain elevated protective memory–antibody levels, one may conclude that the phenotype of immunological memory (enhanced protection against reinfection and transferable protection for offspring) is not a special quality of the immune system but represents equilibrated, long-term, low-level responses driven by some forms of persisting antigen.

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ANTIGEN PROCESSING AND PRESENTATION BY THE CLASS I MAJOR HISTOCOMPATIBILITY COMPLEX[1]

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KEY WORDS: class I major histocompatibility complex, antigen processing, antigen presentation, cytotoxic T lymphocyte, antigenic peptide

ABSTRACT

Major histocompatibility complex (MHC) class I molecules bind peptides derived from cellular proteins and display them for surveillance by the immune system. These peptide-binding molecules are composed of a heavy chain, containing an antigen-binding groove, which is tightly associated with a light chain (β_2-microglobulin). The majority of presented peptides are generated by degradation of proteins in the cytoplasm, in many cases by a large multicatalytic proteolytic particle, the proteasome. Two β-subunits of the proteasome, LMP2 and LMP7, are inducible by interferon-γ and alter the catalytic activities of this particle, enhancing the presentation of at least some antigens. After production of the peptide in the cytosol, it is transported across the endoplasmic reticulum (ER) membrane in an ATP-dependent manner by TAP (transporter associated with antigen presentation), a member of the ATP-binding cassette family of transport proteins. There are minor pathways for generating presented peptides directly in the ER, and some evidence suggests that peptides may be further trimmed in this location. The class I heavy chain and β_2-microglobulin are cotranslationally translocated into the endoplasmic reticulum where their assembly may be

[1]Abbreviations: MHC I, class I major histocompatibility complex; CTL, cytotoxic T lymphocyte; IFN, interferon; HC, heavy chain; β_2-m, β_2-microglobulin; ER, endoplasmic reticulum; HSP, heat shock protein TAP, transporter associated with antigen presentation.

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facilitated by the sequential association of the heavy chain with chaperone proteins BiP and calnexin. The class I molecule then associates with the lumenal face of TAP where it is retained, presumably awaiting a peptide. After the class I molecule binds a peptide, it is released for exocytosis to the cell surface where cytotoxic T lymphocytes examine it for peptides derived from foreign proteins.

INTRODUCTION

Tumors and viral infection present a special problem in recognition for the immune system because the etiologic agents (virus or dysregulated oncogenes) are hidden inside autologous cells. Moreover, humoral immune mechanisms such as antibody and complement are generally ineffective against tumors or intracellular viruses. These problems have been solved by the class I major histocompatibility complex (MHC I) antigen presentation pathway, which is constitutively active in most somatic cells and upregulated by proinflammatory cytokines. In this pathway, fragments of cellular proteins are bound by MHC I molecules and transported to the cell surface. In this manner cells display a sampling of their expressed genes. Usually only autologous peptides are present on MHC I molecules, and these are ignored by the immune system. However, if a cell is synthesizing a foreign protein (which in nature only occurs when cells are virally infected or have mutated or dysregulated the expression of their own genes), then immune effector cells will be stimulated.

The effector cell in this system is a $CD8^+$ cytotoxic T lymphocyte (CTL). During development in the thymus, CTL precursors bearing T cell receptors that can recognize self–MHC I molecules with foreign peptides mature while those cells that react with self-peptides are eliminated (1). As a consequence of this mechanism, mature CTL are highly specific for foreign peptides bound to MHC I molecules. These cells continuously recirculate through the body. If a CTL happens to contact a cell expressing the MHC I/foreign antigen complex for which it is specific, signals transduced through the T cell receptor cause the CTL to become activated, rapidly setting into motion both secretory and nonsecretory cytotoxic machinery (2). In addition, CTLs produce cytokines such as interferon-γ (IFN-γ), which induce inflammation, further stimulate antigen presentation, and may also interrupt a viral replication cycle without killing the target cell.

Experiments with adoptive transfer or in vivo depletion of lymphocyte subsets have shown that $CD8^+$ cells alone can clear some (3–5), though not all (6), viral infections. Consistent with these results, mutant mice lacking CTL are more susceptible to some but not all viruses (7). However, human patients deficient in MHC I antigen presentation do not appear to have a higher incidence of severe viral infections (8). The ability to resist or clear viral infections without $CD8^+$

T cells does not necessarily mean that CTL are unimportant, but more likely shows that the immune system has multiple redundant effector mechanisms.

Although there have been several excellent recent reviews on the MHC I antigen presentation pathway (e.g. 9–11), the field has continued to move rapidly. In this chapter, we have tried to emphasize recent progress. Our review is focused on the presentation of endogenous antigens on MHC Ia molecules. Some related issues such as viral evasion of this pathway, the presentation of exogenous antigen (which will be reviewed elsewhere), and the functions of the nonclassical MHC Ib molecules (12) are not covered.

The Structure of Class I MHC Molecules

Mature MHC I complexes consist of three noncovalently associated components: a 45-kDa heavy chain (HC); a 12-kDa light chain, β_2-microglobulin (β_2-m); and a short peptide antigen. The HC are encoded in the MHC locus (chromosome 6 in humans and chromosome 17 in mice). There are three class Ia loci—in humans, called HLA-A, B, and C; in mice, H-2K, H-2D, and H-2L. There are also many class Ib (12) and MIC (MHC class I-chain related) (13) genes that are not covered in this review. The class Ia HC genes are extremely polymorphic (14). Both alleles of each locus are codominantly expressed, so that an individual may express up to six different class Ia molecules. These genes encode type I glycoproteins of approximately 340 amino acids, consisting of a cytoplasmic region (about 30 residues), a transmembrane region, and an extracellular region composed of three domains (α1, α2, and α3) with one to three N-linked glycosylation sites.

β_2-m is a nonglycosylated protein of about 100 amino acids encoded on chromosome 15 in humans and 2 in mice. Only one allele is known in humans, while there are seven in mice. There appears to be little functional significance of the polymorphisms in β_2-m, although they may subtly affect class I structure or peptide binding because different β_2-m alleles can alter CTL recognition of some antigens (15). β_2-m has no transmembrane domain and remains associated with cells by interacting with the extracellular region of HC.

The structure of a number of MHC I molecules has been solved (16, 17; summarized in 18, 19). β_2-m makes extensive contacts with all three domains of the HC (16). Because of this, the conformation of the HC is very dependent on the presence or absence of β_2-m. The membrane-proximal α3 region of the HC is an immunoglobulin-like domain that contains a binding site for the CD8 receptor on CTL. The α1 and α2 domains, which are distal to the membrane and interact with the T cell receptor on CTL, fold together to form a groove that binds and displays peptides. A beta-pleated sheet forms the base of the cleft, and the walls are made of two alpha helices. The allelic polymorphisms in HC primarily occur in those residues in and around this cleft, and in this manner, they

alter the peptide-binding specificity of the class I molecules (14, 17). This is the primary molecular basis of the way in which MHC I genes determine whether an individual can respond to particular antigenic determinants (Ir gene effects).

The solution of the crystal structure of the class I proteins also provided insight into the perplexing problem of how a single type of molecule could bind and present so many different peptides. Many of the molecular interactions are with the peptide's main chain atoms and amino and carboxy termini, which are features common to all peptides. In addition a limited number of peptide side-chains extend into pockets along the groove thus imparting some specificity to peptide binding, although many different peptides can be accommodated (18), and determine general motifs of the peptides capable of binding to particular MHC I haplotypes (20; reviewed in 21). The groove is generally long enough to accommodate 8 or 9 residues in an extended conformation (22). Longer peptides can also fit by bulging partly out of the groove or by zigzagging within the cleft (23). In some cases the ends of the peptide may extend out of the groove, although this decreases the stability of interaction (24, 25). While such longer peptides are identified less often than octa- or nonapeptides, they are a significant minority—in some cases about 20–40% of the total (26).

GENERATION OF THE PEPTIDE EPITOPE

Antigens and Subcellular Location of Antigen Processing

Early studies indicated that class I–associated peptides could be derived from viral antigens resident in the cytosol (27–29), and from proteins that were artificially introduced into the cytosol (30, 31). Moreover, inhibiting protein breakdown in lysosomes did not affect MHC I presentation (32), implying that class I–presented peptides were generated by neutral proteases in an extralysosomal pathway. Together, these data implied that at least some class I–presented peptides were generated in the cytosol. That this was the major location in which antigenic peptides were produced was indicated by the analysis of mutant cell lines that lacked a transporter (TAP: see section on Transport Across the ER Membrane), which shuttled peptides from the cytosol into the endoplasmic reticulum (ER). In these cells there were few peptides available for presentation, indicating that most presented peptides came from the cytosol.

Proteins targeted into the ER by signal sequences can also be presented on MHC I molecules. Since these molecules are cotranslationally transported into the endoplasmic reticulum, they might be expected to bypass hydrolysis in the cytosol. However, where analyzed, the presentation of most of these antigens is dependent on the TAP-transporter (see Transport Across ER Membrane) (summarized in 33) and on proteasome activity (MT Michalek, KL Rock, in

preparation), and therefore the presented peptides are probably being generated in the cytosol. In these cases the immune system may be sampling those molecules that fail to enter the endoplasmic reticulum or fragments of these molecules generated in the ER and exported back into the cytosol (see section on Transport Across ER Membrane). There are a few examples where proteins with signal sequences are presented in TAP-deficient cells (34, 35), and in these cases the peptides are presumably generated in the ER (see section on Transport Across the ER Membrane).

Peptides are also presented from nuclear (28, 36) and mitochondrial proteins (37). Again, where examined presentation of these antigens requires the TAP transporter (38, 39), suggesting that the peptides are present in the cytosol. In these cases it is unknown whether the presented peptides are produced in the nucleus or mitochondria and transferred into the cytosol, or whether they are actually generated in the cytosol.

There is only limited information on how the structure of an antigen influences its presentation. Some studies have examined the effects of changing the sequences flanking an epitope or varying its location in a protein. In some cases these alterations have no measurable effect (40), while in other cases they decrease presentation (41), presumably owing to altered cleavage patterns by proteases (42).

The Ubiquitin Proteasome Pathway

The major pathway for degrading proteins in the cytosol is ATP-dependent and highly conserved from yeast to mammals (43). In this process proteins are hydrolyzed to oligopeptides by a large multicatalytic proteolytic particle, the proteasome (44, 45). One form of the proteasome is a 20S (700 kDa) cylindrical particle composed of four rings (46). In mammalian cells, the outer rings are made up of seven distinct α subunits, thought to be primarily structural and regulatory, while the inner rings are composed of seven distinct β subunits that form the catalytic sites (46, 47). Proteolysis occurs in the aqueous core of this cylinder, an arrangement that presumably protects many cell constituents from unregulated degradation. While the 20S proteasome can degrade some proteins in cell-free systems, it is uncertain to what extent, if any, this complex by itself hydrolyzes proteins in living cells. Another form of the proteasome is a 26S (1500 kDa) particle, which contains as its proteolytic core the 20S structure associated with many additional subunits that regulate its activity (44, 45). This larger particle degrades some protein substrates directly in an ATP-dependent manner. In addition, the 26S structure binds polyubiquitin chains (48) and is responsible for degrading ubiquitin-conjugated proteins.

The degradation of many cellular proteins is initiated by their modification with the small polypeptide ubiquitin (49). Ubiquitin is attached via an

isopeptide bond to lysines on the substrate and on other ubiquitins. This process requires ATP and several enzymes (E1, E2, and E3) that activate the ubiquitin and transfer it to the protein substrate. The polyubiquitin chain serves as a molecular tag that marks a protein for rapid degradation by the 26S proteasome. The specificity of this process is determined by the E2 and/or E3 enzymes which bind to specific substrates and then conjugate ubiquitin.

In both yeast and mammalian cells, the degradation of short-lived normal and abnormal proteins was markedly impaired by mutations in the ubiquitin activating enzyme, E1, which inhibit ubiquitin conjugation (50). A similar phenotype was seen in yeast with a mutation in a proteasome subunit (51). More recently, peptide aldehyde inhibitors of the proteasome were found to block the degradation of not only short-lived proteins but also the majority of long-lived proteins that constitute the bulk of cell proteins (52). Since the proteasome pathway is in the cytosol and appears to be responsible for the extralysosomal degradation of the majority of cell proteins, it was a candidate for generating class I–presented peptides (53). Interest in this possibility increased with the discovery that two subunits of the proteasome were encoded in the MHC region of the genome (Section III, E).

The Role of Ubiquitin in Antigen Presentation

Initial experiments to analyze the role of the ubiquitin-proteasome pathway in antigen presentation examined the importance of ubiquitination. The presentation of native ovalbumin injected into an E1 temperature-sensitive (TS) mutant cell line was significantly inhibited at the nonpermissive temperature. In contrast, if the need for proteolysis was bypassed by expressing the relevant ovalbumin peptide directly in the cytosol, then presentation occurred normally, indicating a role for ubiquitination in antigen processing (54). This role of ubiquitin in ovalbumin presentation was recently questioned by Cox and coworkers, who found that inhibition at the nonpermissive temperature in TS cells was observed in only two of three experiments with injected ovalbumin, and not at all when ovalbumin was expressed from vaccinia virus (55). However, several technical differences between these studies may account for the differences observed. Moreover, the ovalbumin that is available for presentation from vaccinia is presumably misfolded in the cytosol, and we have found that this antigen when denatured is presented in TS mutants at nonpermissive temperature due to residual E1 activity and/or a ubiquitin-independent pathway (MT Michalek, KL Rock, in preparation). A similar defect was not observed in the presentation of several other antigens by TS cells (55). However these negative results are not interpretable because ubiquitin conjugation cannot be completely inhibited in these cells. Unfortunately, this is a major limitation of the TS cell lines.

Other studies have tested the effect on presentation of enhancing the ubiquitination of an antigen. This was accomplished by attaching to proteins an amino-terminal sequence (the "N-end rule") that is recognized by ubiquitin conjugating enzymes and should lead to ubiquitin conjugation (56). These modifications led to more rapid presentation of peptides from influenza nucleoprotein (57) and β-galactosidase (58). In the case of β-galactosidase, it was confirmed that enhanced ubiquitin-dependent degradation of the substrate was induced and was responsible for the effects on antigen presentation (58).

Thus at least for selected model antigens, ubiquitin conjugation plays a role in MHC I presentation. An important conclusion from these experiments is that protein degradation is a rate-limiting step for class I presentation; whether this will be true for antigens that are intrinsically less stable than β-galactosidase remains to be determined. There is also limited evidence that under some circumstances, e.g. with denatured and chemically modified ovalbumin, ubiquitin-independent presentation can also occur (MT Michalek, KL Rock, in preparation). A major unresolved question is whether ubiquitination plays a role in the presentation of other antigens.

The Role of the Proteasome in Antigen Presentation

The role of ubiquitin in antigen presentation indirectly implicates the proteasome as the protease responsible for generating presented peptides. Although the oligopeptide products from the proteasome have not been extensively analyzed, limited data suggest that the products are typically between five and eleven amino acids long (59, 60), possibly due to the distances between the proteasome's active sites (46). Interestingly, this is the size range for peptides that is optimal for binding to different MHC I molecules. Several studies have shown that purified proteasomes in vitro can generate class I–presented peptides from oligopeptide substrates (42, 61) and from intact antigens such as ovalbumin and β-galactosidase (62), although these experiments have used only 20S particles and nonphysiological conditions. These experiments show that the proteasome is capable of generating presented peptides, although they do not prove that it does so in vivo.

Direct evidence in vivo for a role of this particle in antigen presentation was obtained by blocking its function with tripeptide and dipeptide aldehydes. These agents inhibit the class I presentation of protein antigens [ovalbumin (52, 63) and β-galactosidase (58)], without affecting presentation of the relevant peptides expressed or injected into the cytoplasm (52). The interpretation of these experiments rests on whether the effects of the peptide aldehydes on antigen presentation are due to inhibition of the proteasome. This seems to be the case, since for the seven agents that have been tested, their potency for blocking antigen presentation is closely correlated with their potency in

inhibiting the proteasome over a several log range in Ki's but is unrelated to their effects on other known proteases. Furthermore, antigen presentation is also blocked by highly selective proteasome inhibitors of very different structure (47; A Craiu, G Fenteany, SL Schreiber, KL Rock, unpublished data). Further independent support for a role of the proteasome in antigen presentation was obtained in analyses of proteasome LMP subunits described below.

Is the proteasome the major pathway for generating class I presented peptides? The effect of proteasome inhibitors on overall peptide production has been assayed indirectly. These agents do not block the synthesis of MHC I heavy or light chain but markedly inhibit the stable assembly of these two chains; this block in assembly can be overcome by adding exogenous antigenic peptides (52). Since the stable assembly of the class I heterodimer is dependent on the binding of peptides (see section on Peptide Processing in the Endoplasmic Reticulum) these results suggest that the proteasome plays a major role in supplying peptides to class I molecules. The block in assembly is not complete, leaving open the possibility that there are other pathways that contribute peptides. Peptides cleaved by proteases other than the proteasome—including peptides derived from signal sequences cleaved by the signal peptidase—can also be presented via MHC I (64, 65) so long as they contain the appropriate binding motifs. Furthermore, it is not clear whether the peptides produced by the proteasome are identical to those bound by MHC I, or whether they undergo further processing in the cytosol or in the ER (Section V), and if the peptide does undergo further processing, whether the proteasome is responsible for the N- or C-terminal cleavage.

The MHC I is apparently indifferent to the source of peptides. In fact, proteolysis is not required for entry into the class I antigen presentation pathway in all situations. Peptides expressed from minigenes—genes that encode only the MHC-binding epitope of interest—are also presented efficiently (66). It has also been proposed that aberrant transcription or translation may generate short peptides (peptons) which get presented on MHC I without further proteolysis. While some oligopeptides are probably generated through these mechanisms (67), available evidence suggests that this is a minor source of class I–presented peptides and/or that these peptides require further proteolysis (52, 68).

LMP Subunits and Regulation of Proteasome Activity

Among the first data pointing to the potential role of the proteasome in antigen presentation was the discovery that two genes in the MHC (LMP2 and LMP7) encoded subunits of the proteasome (69, 70). LMP2 and LMP7 are members of the β subunit family of proteasome subunits. Although most cells constitutively express low levels of these components, IFN-γ upregulates their expression (71) and posttranscriptionally downregulates their constitutively expressed

homologues, so that IFN-treated cells develop a subpopulation of proteasomes containing LMP2 and LMP7 (72–74).

To investigate the function of LMPs, cells that lacked these subunits were tested for their ability to present antigens. To develop such cells, a mutagenized human cell line (721.174 and its derivative, T2), which had a large deletion of MHC genes (75, 76), was transfected with TAP (peptide transporter) genes. These LMP2- and LMP7-deficient cells were initially found to have no qualitative or quantitative defect in antigen presentation (77–79). Recently, however, it has been shown that this cell is impaired in its ability to present some antigens and that some of these defects can be corrected by transfection of LMP7 (80). While these data indicate that LMPs are not essential and may play a relatively minor role, caution is needed in generalizing results from a single transformed and transfected cell line.

Stronger evidence for a role of LMP2 and LMP7 was seen with mutant mice, lacking either LMP2 (81) or LMP7 (82). In the LMP7 knockout mice, the level of cell-surface MHC I was moderately reduced, to between 55% and 90% of normal, which is consistent with a deficiency in the supply of antigenic peptides. Moreover, presentation of at least one antigen (H-Y) was impaired. Although the LMP2 mutant mice did not show a reduction in cell-surface MHC I, they had a reduction in $CD8^+$ T cells (possibly due to an alteration in the peptides available to select these T cells in the thymus) and moderately impaired responses both in vitro and in vivo to some, though not all, viral antigens (81). In the mutant mice the possibility has not been fully excluded that homologous recombination of the LMPs has affected the expression of the closely linked TAP genes (known to be essential for antigen presentation; see Transport Across the ER Membrane). However, this is unlikely because TAP mRNAs are not reduced in LMP7 or LMP2 mutant mice (82; L Van Kaer, personal communication), and the LMP2 mice present some antigens normally (81). Mice lacking both LMP2 and LMP7 have not yet been produced and will be needed to determine whether LMP subunits play a major or minor role in antigen presentation. Nevertheless, these findings provide genetic evidence linking the proteasome to class I antigen presentation.

How do the LMP subunits influence antigen presentation? Proteasomes purified from cells lacking LMP2 and LMP7 show a different pattern of catalytic activities than do those derived from LMP2- and LMP7-expressing cells. This effect is almost certainly due to the LMPs participating in the active sites of the proteasome. Consistent with this interpretation, both LMP2 and LMP7 have an amino-terminal threonine, a residue that has been identified as the active site nucleophile for proteasomes from archebacteria and mammals (47, 83). Compared to LMP^- proteasomes, LMP^+ proteasomes cleave peptide

substrates more rapidly after basic and hydrophobic residues (81, 84–87), while cleavage after acidic residues is unchanged (84) or reduced (85, 86) in most but not all studies (61, 88). Therefore LMPs are predicted to alter the pattern of cleavages in antigens, favoring the generation of immunogenic peptides. In fact, proteasomes from IFN-stimulated cells generated the appropriate epitope from a 25mer peptide containing a nonameric peptide epitope, while proteasomes from unstimulated cells cleaved a site within the nonamer and destroyed that epitope (61). One model for how the generation of antigenic peptides might be favored is that the changes in peptidase activities are predicted to lead to the generation of more peptides that terminate in hydrophobic and basic residues and fewer with acidic ones (84, 85). This pattern might be significant because peptides bound to MHC I almost invariably have basic or hydrophobic C-termini (89). However, it may be that the alterations in catalytic activities lead to more immunogenic peptides in more complex ways.

TRANSPORT ACROSS THE ER MEMBRANE

TAP and ABC Transporters

Peptides produced in the cytosol must pass into the lumen of the ER before they can bind to MHC I molecules that are assembled in this organelle. The first indication that this transfer into the ER required a specific transporter came from mutant cell lines that had low cell-surface expression of MHC I and were deficient in antigen presentation. These cells synthesized heavy and light chains normally, but the chains did not stably assemble (90) (due to a deficiency in available peptides—see section on Assembly). That the defect in these cells was in the transfer of peptides into the ER was suggested when it was found that the defect in MHC I expression and antigen presentation was corrected simply by exposing the cells to an antigenic peptide in the extracellular fluids (90). These experiments provided the field with a key conceptual insight, although the mechanism by which the exogenous peptides normalized the mutant phenotype was probably not by retrograde transport into the ER, as originally considered, but by stabilizing class I molecules at the cell surface (91, 92). Further support for this model came from the observation that peptides directed into the ER by a signal sequence were presented on MHC I in the mutant cells (66).

The genes responsible for this effect were tentatively mapped to the MHC locus by analysis of gene deletions in one of these mutant cells (93). Several groups cloning genes from the MHC region for various reasons identified genes homologous to members of the ATP-binding cassette (ABC) family of transport proteins (94, 95). These genes are now called TAP (transporter associated with antigen presentation), and their role in antigen presentation was confirmed when

transfection of the genes corrected the antigen-presentation defect in the mutant cell lines (94, 95). As expected, mutant mice lacking TAP1 (96) and human patients lacking TAP2 (8) show profound defects in MHC I-restricted antigen presentation.

ABC transporters are found in both prokaryotes and eukaryotes (97). The members of this family transport across cell membranes a wide range of molecules, ranging from ions to large proteins. They have either 6 or 12 membrane-spanning domains; those with 6 transmembrane domains, such as the TAP proteins, function as heterodimers or homodimers. The two subunits of TAP are approximately 76 (TAP1) and 70 (TAP2) kDa and are noncovalently associated. Although one study suggests that TAP1 might also be able to function as a homodimer (98), TAP1 and TAP2 generally seem nonfunctional when expressed alone (99, 99a). Immunoelectron microscopy and confocal analysis have localized TAP to the ER (99a) and the *cis*-Golgi (100). This subcellular distribution is consistent with the location in cells where MHC molecules bind peptides (see Assembly).

Transport of Peptides Across the ER Membrane

Since TAP mutant cells appeared to have a defect in supplying peptides to class I molecules and the TAP genes were homologous to known transporters, it seemed extremely likely that TAP functioned to transport peptides from the cytosol into the ER. However, initial attempts to analyze peptide transport in microsomes derived from TAP^+ and TAP^- cell lines complicated the issue somewhat, as it seemed that transport was neither ATP nor TAP dependent (101–103). More recent studies, however, have found that transport of peptides into microsomes, or across the ER membrane of permeabilized cells is TAP- and ATP-dependent (104–106).

The sequence of both the TAP1 and TAP2 subunits shows consensus ATP-binding sites in the cytoplasmic domain, near the C-terminus. ATP (and other nucleotides) binds to both these sites (99a, 107, 108) in a $Mg2^+$ (and, to a lesser extent, $Ca2^+$) dependent manner; this is probably due to a $Mg2^+$-binding site in the ATP-binding region (99a). The reason for the effect of $Ca2^+$ is not known. The peptide-binding site is also in the cytoplasmic region and is probably formed by both subunits (109, 110). Peptide binds to the TAP complex independently of ATP (109), while peptide translocation is ATP-dependent (104).

The profound defect in antigen presentation in cells lacking TAP function indicated that this transporter is responsible for supplying most peptides for class I presentation, and it therefore must have very broad specificity. However, early studies also make clear that the transporter would have some selectivity because a genetic polymorphism in the TAP genes of rats affected the repertoire of presented peptides (111). Recent studies have begun to analyze the specificity

of TAP in transport assays using microsomes or permeabilized cells. Most of these studies have determined TAP preferences based on competition assays, which probably are most affected by peptide binding; there may be additional constraints on translocation. Moreover, not all studies have controlled for the possibility that peptides might be trimmed by peptidases prior to TAP transport. With these caveats, the transporters have definite preferences for peptides of a certain length, and less dramatic sequence preferences (112, 113).

TAP only transports peptides longer than 7 residues; its efficiency drops off dramatically with peptides longer than about 12 aa, but there is no clear upper limit (104, 106, 114): This is the general range that is optimal for peptide binding to MHC molecules. There is also some specificity for a peptide's C-terminal residue. In mice, MHC I molecules show preferences for binding peptides containing hydrophobic residues at the C-terminus, and human MHC I mainly binds peptides with hydrophobic and basic C-termini; the TAP transporters in each case show a similar bias (115, 116). In rats different TAP2 alleles show specificity similar to either the human or the mouse TAP (116, 117). The preferences for other residues or sequences are not yet well defined, although it is clear that in some cases they can also affect the efficiency of translocation (117, 118). Other than in rats, polymorphisms in TAP do not seem to have a major effect on their specificities for peptides (119).

One complicating factor in analyzing TAP-dependent transport is the presence of an efficient ATP-dependent peptide export system directing peptides out of the ER and into the cytoplasm (115, 120). This transport activity does not appear to be mediated by TAP but by some as yet unidentified mechanism. Little is known about this system's specificity (if any) or capacity, but it seems easily able to keep up with import by the TAP transporters. Possibly this peptide exporter selects for high-affinity peptides, in that those of low affinity are not rapidly bound to the MHC I and are therefore exported. It might also subserve nonimmunological roles in cells such as transferring the products of ER degradation to the cytosol for further hydrolysis.

Some peptides that are expressed in the cytosol of cells lacking TAP transporters are presented on MHC I (121). This suggests a TAP-independent mechanism for transferring peptides into the ER. Since there are few class I presented peptides in TAP mutant cells, however, this must be a minor pathway.

Other Aspects of Peptide Transport

Are other, as yet unknown, proteins required for TAP function? Since TAP expressed in insect cells (i.e. in the absence of an antigen presentation system) appears to be functional (99), proteins limited to the vertebrate immune system are probably not essential. Similarly, in cell-free systems, no cytosolic fraction is required for specific TAP-dependent transfer across microsome membranes,

although this does not mean that accessory proteins could not improve the efficiency of transport. However, at 37°C, peptide binding in insect cells (109) and translocation in microsomes is inefficient (99a, 106), so there may be other factors in mammalian cells that influence TAP function. Interestingly, several as yet unidentified proteins coimmunoprecipitate with TAP, particularly when peptide transport is blocked by the viral protein ICP47 (122; P Jugovic, DC Johnson, personal communication).

Since peptides are very rapidly degraded in the cytoplasm (114, 123), it has been proposed that peptides may associate with chaperone proteins [such as members of the heat-shock protein (HSP) family] as they exit the proteasome, protecting and guiding the peptides to the TAP, while other chaperones protect the peptides in the lumen of the ER until they can interact with the MHC. Several HSP70 genes are encoded in the MHC (124); however, their role in antigen presentation, if any, is unknown. Peptides can become associated with members of the HSP family in the cytoplasm, ER, and mitochondria; this is not unexpected because HSP bind to a wide range of peptides and proteins (125). Therefore, the finding of antigenic peptides bound to HSP molecules does not in and of itself prove an immunological function.

The only data that suggest that such complexes can supply peptides to class I are indirect. Immunization of mice with HSP (cytosolic, mitochondrial, or ER-derived) purified from tumors can stimulate antitumor-specific CTLs. Although this appears to be due to the antigenic peptides bound to the HSPs (summarized in 126), the mechanism by which the chaperones in the extracellular fluids donate peptides for class I presentation is not known and may be unrelated to normal events in the endogenous class I pathway. In summary there is presently no firm evidence that chaperones play a role in a role in shuttling peptides to and from the TAP transporters.

Another interesting possibility is that the proteasome might transiently interact with TAP, delivering peptides directly to the transporter. There is as yet no evidence for this, and since peptides artificially delivered to the cytoplasm or expressed by minigenes are presented, a linkage between TAP and proteasomes is not essential for transport. Again, though, such an interaction could increase the efficiency of peptide transport.

PEPTIDE PROCESSING IN THE ENDOPLASMIC RETICULUM

Some proteins, such as incompletely assembled receptors, are hydrolyzed in the ER by an as yet poorly characterized degradative pathway (127). This is a potential source of peptides for class I presentation, and there are a few

examples in TAP mutant cells of antigens presented on MHC I after they are imported into the ER by signal sequences. However, the paucity of presented peptides in TAP-deficient cells indicates that this is a not a major pathway for generating peptides in their final form (33, 130). An important unresolved issue is whether most peptides generated in the cytosol are presented without further modification or are produced by trimming of longer peptides in the ER. TAP can transport peptides that are longer than the optimal length for binding to MHC I molecules. In some cases, these peptides do bind to MHC I molecules (see The Structure of Class I MHC I Molecules). However, in other cases only peptides of optimal length are presented despite the transport of longer species which can also bind (128). In yet other cases, authentic MHC I–binding peptides are transported by TAP much less efficiently than are slightly longer versions of the peptide (118). In addition to the possibility of trimming in the ER, it is possible that long peptides could be transported out of the ER by the peptide efflux pump, be further processed in the cytoplasm, and then re-enter the ER through TAP (120).

Several experiments have suggested that peptides can be further processed after being transported into the ER. By combining sequentially in a minigene a signal sequence followed by two antigenic peptides, Snyder and coworkers (129) found that in TAP-mutant cells the C-terminal epitope was presented well while the N-terminal one was presented poorly or not at all, suggesting that an aminopeptidase activity in the ER could trim the peptides. A similar experiment demonstrated amino-terminal trimming of an influenza nucleoprotein peptide (130). However, neither of these studies excluded the possibility that this "trimming" of the peptides was due to an imprecise cleavage by the signal sequence peptidase. These studies did suggest that the ER does not contain an efficient mechanism to trim the C-terminus of at least some peptides because the amino-terminal epitope in one of these constructs was presented poorly. Similarly, a peptide that was extended by two carboxyterminal residues was not presented when expressed in cells unless it was coexpressed with a carboxypeptidase that was transported into the ER (65).

One possibility is that MHC I molecules first bind long peptides, but that peptidases trim off the overhangs, leaving the portion protected by the MHC I groove intact (131). Since many MHC I molecules may reach the cell surface with long peptides (89), trimming of overhangs is inefficient at best. Moreover, this is unlikely to account for the majority of properly fitting peptides, since a one-residue overhang is probably too close to the MHC to accommodate a protease active site (25). Furthermore, long peptides that are bound to MHC I molecules appear to be protected from digestion by proteases in cell-free systems (132). Therefore, if trimming occurs it seems more likely to take place

before peptides bind to MHC molecules. However, if the physical interaction of MHC I with TAP is normally important in the charging of class I with peptide, then there may be little opportunity to trim peptides before binding.

Based on available data, it is difficult to estimate the importance of peptide trimming in the ER. Since the proteasome may well produce peptides mainly of about eight amino acids (60), it remains possible that most presented peptides will not require trimming. Even if this is not the case, the bias for peptides optimal length could simply arise from selection for the highest affinity peptides that are transported into the ER even if they are of lower abundance. Further experiments will be needed to resolve these issues.

ASSEMBLY

Subcellular Location and Order of Assembly of MHC I Molecules

The MHC I heavy and light chains are synthesized separately on the rough ER and are cotranslationally transported into the ER. To be efficiently transported to the cell surface and displayed to the immune system, HC, β_2-m, and a peptide must assemble together. Several lines of evidence suggest that assembly occurs in a pre-Golgi compartment, probably the ER itself. N-linked oligosaccharides on HC in mutant cell lines lacking TAP (which do not efficiently translocate peptides into the ER) are not processed by the enzymes in the *cis* and medial Golgi (133, 134). Therefore, peptide binding must normally occur in or before the *cis*-Golgi compartment. This is supported by the observation that MHC I bound to the adenovirus protein E3gp19k (which binds to some MHC I complexes and causes their retention in the ER) are loaded with peptides in vivo (135). Similarly, MHC I in cells treated with brefeldin A, which prevents glycoproteins from exiting the ER, are still able to bind peptide (136).

The assembly of MHC I molecules could proceed through two different pathways: HC and β_2-m associate, and then peptide is added; or peptide and HC may associate, followed by β_2-m (137). Both potential intermediates (HC/β_2-m and HC/peptide) are much less stable than the mature complex (138, 139). In vitro, both pathways can produce a mature complex (138, 140), but the former may be the major pathway in normal cells (141–143). In cells lacking β_2-m, which can only use the second pathway, only a very small amount of HC becomes correctly folded (and therefore, probably, peptide-associated) at physiological temperature (144), whereas in cells that lack peptide, HC/β_2-m heterodimers, although still relatively unstable at physiological temperature, are readily detectable in the ER (96, 145). Moreover, most empty class I heterodimers in the ER are bound to TAP, whereas heavy chains alone are not (see Transport of Peptides Across the ER Membrane). Therefore if most class

I molecules acquire their peptide when bound to TAP, as seems likely, then HC/peptide intermediates are probably a minor pathway.

Interaction of Newly Synthesized MHC I with Chaperones and TAP

The assembly process may be facilitated by the formation of a series of intermolecular interactions (146): The immature HC or the HC/β_2-m complex is associated with ER chaperone proteins, and the HC/β_2-m complex then associates with TAP until peptide binds. Until all three components of the complex are present, the HC and peptide are retained within the ER. The rate of assembly varies widely in different cell types and cell lines, and different alleles can also have very different rates of assembly and transport (147).

The first chaperone to associate with HC is BiP. A population of human, but apparently not mouse, HC associates with BiP in the ER (148, 149). BiP (GRP78), a member of the HSP 70 family, is an ER chaperone involved in the folding and retention in the ER of many proteins (150). Although in general BiP associates with incompletely folded proteins, the details and the importance of the interactions are not well understood. At least some of the HC associated with BiP is able to associate with β_2-m (148), suggesting that this might not be not a dead-end pathway.

Calnexin is an ER-resident transmembrane protein involved in the folding, assembly, and retention of many transmembrane glycoproteins and complexes (151). Calnexin has lectin domains and interacts with many substrates through recognition of partially trimmed oligosaccharides (152), and this may also apply to the heavy chain (153). However, this cannot be the sole means either of initial recognition or of stable binding, as enzymatic removal of carbohydrate groups from the HC does not cause the HC/calnexin complex to dissociate (152, 154), and calnexin can still interact with mutated HC that lacks N-glycosylation sites altogether (155). Whether in addition to or instead of recognizing carbohydrates, calnexin apparently binds via protein-protein interactions to the transmembrane region (156) or the connecting peptide immediately adjacent to the membrane (155).

Calnexin binds newly synthesized HC, enhances its folding and assembly, and also reduces its degradation (146, 157). The finding that the kinetics of HC dissociation from calnexin are correlated with the kinetics of transport of MHC I from the ER raised the possibility that calnexin regulates transport (158). However, calnexin dissociates from human HC when the HC/β_2-m complex forms (149, 159), although this complex is still immature and is retained in the ER until peptide is added. While the mouse HC appears to remain associated with calnexin after β_2-m binds (149, 158), and even during the interaction

with TAP (see below), it is not clear that calnexin remains associated until peptide is added (146). On the other hand, in cells lacking some components of the assembly process, such as β_2-m negative cells (160) or transfected insect cells (lacking TAP and perhaps other factors)(157), calnexin does retain the incomplete complex in the ER. Nevertheless, since a mutant cell line lacking calnexin shows apparently normal assembly and expression of MHC I (161), calnexin probably is not essential for MHC I folding and assembly.

The last protein with which MHC is known to be complexed is TAP (162, 163); the interaction seems to involve the luminal domains of the HC (W-K Suh, Y Yang, PA Peterson, DB Williams, personal communication) and TAP1 (164). In a mutant cell line in which TAP and MHC fail to associate, peptide does not complex with MHC (164a). Presumably the interaction facilitates peptide association with the HC/β_2-m complex by holding the heterodimer in a region of high peptide concentration, or perhaps by directly delivering peptide to the class I molecule. It is also possible that this interaction with TAP enhances folding of the class I molecule, because there are more peptide-receptive complexes in TAP^+ cells than in TAP^- cells even though the former also have many more peptide-containing complexes (165). Artificially delivering peptides to the cytoplasm resulted in more rapid dissociation of MHC I from TAP, but only when the peptide would bind to the particular MHC I complex (163). This suggests that peptide binding releases class I from TAP. Therefore, TAP is an obvious candidate for retaining in the ER HC/β_2-m complexes lacking peptide.

The order of assembly in vivo, then, may be that the HC associates with BiP (in humans) and then with calnexin, which prevents degradation, facilitates appropriate folding, and either permits or assists with β_2-m assembly. The dimeric complex is then transferred to TAP, which may in turn assist with further folding and which also provides peptide. Peptide itself dramatically enhances the stability of the complex and allows release of the trimeric complex from TAP, and the complex is then allowed to leave the ER and reach the surface. Interactions with the chaperone proteins and TAP are not essential for folding but make it more efficient and complete. Moreover, this mechanism reduces the number of empty heterodimers that reach the cell surface, although some can exit the ER (165, 166). These empty heterodimers, however, are unstable and are rapidly denatured and/or degraded after they reach the cell surface.

MHC I AT THE CELL SURFACE

After assembly MHC I molecules transit through the Golgi where N-linked carbohydrates are modified. They are then rapidly transported by the default exocytic pathway to the plasma membrane where they display their bound peptides. There are actually very few MHC I molecules on the cell surface

that are empty (lacking peptides) (167, 168). This is because most empty molecules (lacking peptides) are retained in the ER (see section on Interaction of Newly Synthesized MHC I with Chaperones and TAP). Moreover, when a peptide dissociates from a MHC I molecule already on the cell surface, creating an empty molecule, or when an empty molecule does reach the cell surface, the heterodimer is unstable and rapidly dissociates (166, 167, 169). These mechanisms limit the binding of exogenous peptides and thereby ensure that class I molecules accurately display the antigens a cell itself has synthesized (167).

Under some circumstances MHC I molecules can bind peptides at the cell surface (91, 170, 171). Peptides, particularly optimal sequences at high concentrations, can bind to empty class I molecules before these molecules dissociate (91, 92, 170). Also, empty MHC I molecules can be stabilized to varying extents by incubation at lower temperature (133), by chemical cross-linking (172), or by incubation with exogenous β_2-m (particularly xenogeneic β_2-m, which appears to more stably bind murine HC) (173–176); these empty molecules can bind peptides. Moreover, free heavy chains on the cell surface can rebind β_2-m (which is a constituent of fetal calf serum used in culture) and peptides (173). In some cases, exogenous peptide has been reported to bind to MHC I by exchanging with a bound peptide (177, 178). It is uncertain whether there is significant peptide binding to cell surface MHC I in vivo.

The half-life of MHC I molecules on the cell surface varies considerably depending on the cell type and the particular class I allele. For some MHC I molecules, such as H-2K^k, the half-life can be greater than 20 h (179) while for others, such as H-2L^d, it is about 2 h (although this short half-life may be due to a lack of peptide) (180). An important determinant in the stability of the class I molecule is the rate of dissociation of peptide and β_2-m. When the heterodimer dissociates, the resulting free heavy chains denature and are degraded over time (92, 166, 175).

In most cell types, the trimolecular complex is not constitutively internalized, although multivalent ligands can induce internalization (181). In T lymphocytes (182) and macrophages (183), however, MHC I is spontaneously endocytosed through clathrin-coated pits, an effect mediated by signals in the cytoplasmic portion of the heavy chain (184). The functional significance of this internalization is unclear. Some endocytosed class I molecules are recycled to the cell surface (185) and might conceivably present peptides from endosomes. Internalized MHC I may also be degraded. Consistent with this, the half-life of MHC I on T lymphoblasts is much shorter than on B lymphoblasts, which do not internalize the complex (5 h vs 20 h) (179).

SUMMARY

Over the last decade remarkable progress has occurred in elucidating the MHC I pathway of antigen presentation. There is now a high-resolution picture of MHC I molecules and the peptides that they present. The broad outline of many of the key steps involved in supplying these presented peptides is emerging. Catabolism by the proteasome turns over the bulk of cellular proteins and in so doing provides the class I pathway with one source of peptides for presentation. A peptide transporter of broad specificity, TAP, transfers peptides from the cytosol to empty class I molecules in the ER. The assembly of MHC I molecules, their retention in the ER, and their continued integrity are all dependent on binding peptides, which presumably ensures efficient use of these peptide-binding receptors and more importantly, perhaps, the immunological identity of cells. An emerging theme is that several of these components, such as the class I molecule and the peptide transporter, are linked presumably to increase the efficiency of presentation. Moreover, many steps that are part of a general cellular pathway (e.g. the proteasome) or that can occur alone (e.g. assembly of the MHC I trimolecular complex) are enhanced by specific components of the immune system (LMP2 and 7 in the case of the proteasome, and TAP-binding in the case of assembly).

Many important issues concerning the known steps of the pathway remain to be resolved, including: What features of an antigen determine the extent of its presentation and which particular epitopes are ultimately displayed? What are the various pathways and steps involved in producing the presented peptides, and how are they regulated? How does the class I pathway survey the antigens resident in different subcellular locations? Are there mechanisms for protecting peptides from destruction and increasing their translocation into the ER and binding to MHC I molecules? How do TAP transporters bind and transport peptides, and what is their fine specificity? What role does the association between TAP and MHC I molecules play in assembly of the heterodimer and the transport and loading of peptides?

Beyond what we currently know, are there other key steps in the pathway that remain to be discovered? While this cannot be definitively answered today, we think it likely that the complexity is significantly greater than is currently appreciated. In part this expectation comes from an uncertainty that the remarkable efficiency of MHC I presentation is fully explained by available data. Moreover, there are hints of additional components in the pathway from as yet unexplained defects in mutant antigen presenting cells (164a, 186) and from the apparent association of a number of as yet unidentified proteins with ER-resident MHC I (187) and with TAP (122; DC Johnson, P Jugovic, personal communication). Furthermore, in addition to those components of the pathway

that are unique to the immune system, almost certainly others common to many cell biological processes remain to be elucidated.

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ROLE OF CYTOKINES IN RHEUMATOID ARTHRITIS

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ABSTRACT

Analysis of cytokine mRNA and protein in rheumatoid arthritis tissue revealed that many proinflammatory cytokines such as TNFα, IL-1, IL-6, GM-CSF, and chemokines such as IL-8 are abundant in all patients regardless of therapy. This is compensated to some degree by the increased production of anti-inflammatory cytokines such as IL-10 and TGFβ and cytokine inhibitors such as IL-1ra and soluble TNF-R. However, this upregulation in homeostatic regulatory mechanisms is not sufficient as these are unable to neutralize all the TNFα and IL-1 produced.

In rheumatoid joint cell cultures that spontaneously produce IL-1, TNFα was the major dominant regulator of IL-1. Subsequently, other proinflammatory cytokines were also inhibited if TNFα was neutralized, leading to the new concept that the proinflammatory cytokines were linked in a network with TNFα at its apex. This led to the hypothesis that TNFα was of major importance in rheumatoid arthritis and was a therapeutic target. This hypothesis has been successfully tested in animal models, of, for example, collagen-induced arthritis, and these studies have provided the rationale for clinical trials of anti-TNFα therapy in patients with long-standing rheumatoid arthritis. Several clinical trials using a chimeric anti-TNFα antibody have shown marked clinical benefit, verifying the hypothesis that TNFα is of major importance in rheumatoid arthritis. Retreatment studies have also shown benefit in repeated relapses, indicating that the disease remains TNFα dependent. Overall these studies demonstrate that analysis of cytokine expression and regulation may yield effective therapeutic targets in inflammatory disease.

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INTRODUCTION

Cytokines are local protein mediators, now known to be involved in almost all important biological processes, including cell growth and activation, inflammation, immunity, and differentiation. Thus, it is not surprising that they have a role in an autoimmune disease such as rheumatoid arthritis (RA), in which there is chronic inflammation, with fibrosis and the eventual destruction of cartilage and bone.

As all the cytokines cloned first, such as interferon gamma (IFNγ), interleukin 2 (IL-2), tumor necrosis factor (TNFα), and interleukin 1 (IL-1), were mediators of immunity or of proinflammatory activity, the underlying assumption in the initial investigations into the role of cytokines in RA was that those most abundantly expressed were likely to be pathogenic. Thus, the long-term goal of cytokine analysis in RA was to define new targets for therapy. It is now clear that this early view of the role of cytokines in arthritis was far too simple. We now know that many cytokines with proinflammatory features also have anti-inflammatory aspects (e.g. IFNγ), and that those with mostly anti-immune/inflammatory features, such as transforming growth factor β (TGFβ) or interleukin 10 (IL-10), also have proinflammatory effects and hence are potentially pathogenic.

In this review, we focus on RA because it is the most studied form of arthritis, and we compare it with other forms of arthritis where possible, including animal models. Discussion of the role of cytokines in other autoimmune diseases can be found elsewhere, including books recently published (1, 2). The analysis of cytokine expression and regulation in RA, as described here, identified a therapeutic target TNFα, which led to the successful clinical trials using a monoclonal antibody to TNFα. These in vivo studies have confirmed the usefulness of the in vitro analysis performed on RA synovial membrane cell cultures.

THE PATHOGENESIS OF RHEUMATOID ARTHRITIS

Although the etiology of RA remains elusive, susceptibility factors are evident. Thus, the threefold predominance of RA in women may be attributable to hormonal factors, and the clear-cut genetic contribution in this disease is contained predominantly within the HLA class II locus. On one side of the peptide binding groove of HLA-DR, there is a shared epitope, comprising amino-acids 70-74 of the β chain, which is conserved in the DR1 and DR4 disease-susceptible haplotypes (3). This epitope is present in $> 80\%$ of Caucasian RA patients and is the most important evidence to support the concept that T lymphocyte recognition is important at some stage in the pathogenesis of RA, either in shaping the T

cell receptor (TCR) repertoire or in the presentation of an inducing microbial or autoantigenic peptide.

More recently, other candidate genes including cytokine polymorphisms have been investigated. Although an allele polymorphism in the promoter region of TNFα associated with HLA A1, B8, and DR3 was identified and another in the promoter region of the IL-1α gene, these are *not* associated with RA but are with other autoimmune diseases such as systemic lupus erythematosus (SLE) in the case of the TNFα polymorphism (4, 5) and a severe form of arthritis in children (JRA) in the case of IL-1α (6). The discordance in developing rheumatoid disease between identical twins (7) clearly suggests that nongenetic factors are also important, and infectious agents are the most plausible explanation. However, although many infectious agents have been implicated over the years, from viruses to mycoplasma and mycobacteria, none have yet been reproducibly identified in different laboratories.

The pathology of RA extends throughout the synovial joint (Figure 1), and in severe cases involves many other organs. In contrast to the acellular nature of normal synovial fluid, RA synovial fluid is enriched predominantly with neutrophils, but macrophages, T lymphocytes, and dendritic cells are also present. The increase in cellularity, however, is most obvious in the synovial membrane, which becomes infiltrated by cells recruited from the blood. The lining layer of the joint is increased, from 1–2 cells to 6–8 cells thick, and is comprised mostly of activated macrophages (occasionally referred to as *type-A* synoviocytes) with an underlying layer of fibroblast-like cells (occasionally referred to as *type-B* synoviocytes). The deeper layers within the synovium have follicles of lymphoid cells around vessels as well as lymphocytes scattered between them. Neovascularization is prominent, and there are many activated endothelial cells. The most abundant cells in the synovial membrane are macrophages and T lymphocytes, but plasma cells, dendritic cells, and activated fibroblasts are also found. Many of these cells are activated and express abundant HLA class II and adhesion molecules of relevance in antigen presentation (8-13).

The major site of irreversible tissue damage originates at the junction of the synovium lining the joint capsule with the cartilage and bone, a region often termed the *pannus*, an area rich in macrophages. The cells of the pannus migrate over the underlying cartilage and into the subchondral bone, causing the subsequent erosion of these tissues (14). The destruction of the cartilage seen in rheumatic disease is now considered to be mostly due to the activity of matrix metalloproteinases (MMPs), enzymes produced by activated macrophages and fibroblasts in response to proinflammatory cytokines such as IL-1 and TNFα. These enzymes are synthesizedand secreted as latent molecules, with

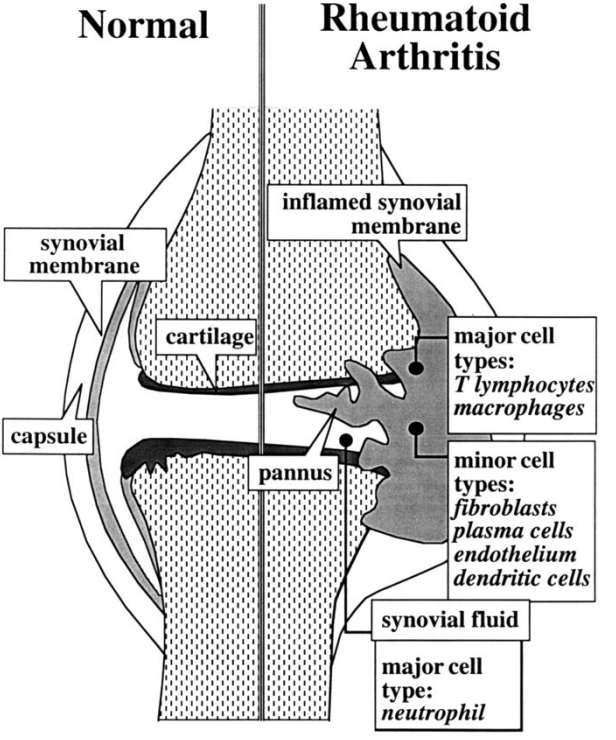

Figure 1 Diagrammatic representation of synovial joint, normal joint (*left*) rheumatoid arthritis joint (*right*).

activation brought about by proteolytic cleavage of the propeptide domain. In arthritis, the MMP enzymes collagenase (MMP-1) and stromelysin 1 (MMP-3), whose production is increased, play an important role in the destructive process (15). The activity of MMPs is regulated to some extent by tissue inhibitors of metalloproteinase (TIMP), three forms of which have been cloned in humans; these irreversibly bind the active MMP to form a 1:1 complex with the enzyme. The fact that the TIMPs are produced by the same cells that produce the MMPs suggests an intimate role for these inhibitors in regulating matrix turnover, and also that much of the connective tissue destruction associated with arthritic disease is due to an imbalance between the production of the MMPs and that of the specific TIMPs. Of interest in this regard is the observation that transforming growth factor β (TGFβ) and IL-10, two immunoregulatory and anti-inflammatory cytokines produced in the RA joint (discussed in the

next section), not only inhibit the production of proinflammatory cytokines that induce MMPs, but also induce the production of their native inhibitors TIMPs (16) (J-M Dayer, personal communication).

In addition to the MMPs, other enzymes synthesized by cells resident within the cartilage and bone as well as by infiltrating inflammatory cells are capable of cleaving the major components of cartilage and bone such as proteoglycan and collagen (type I, II, IX, X, and XI). These enzymes include serine, aspartic, and particularly cysteine endopeptidases such as cathepsin B (17). Detailed discussion of these enzyme effects are beyond the scope of this chapter.

CYTOKINE EXPRESSION IN RHEUMATOID ARTHRITIS

Although the major pathology in RA occurs in the synovium, synovial fluid is more readily accessible, and as such the production of cytokines such as IL-1 was first documented in this compartment (18). However, the relevance of cytokines found in synovial fluid in the pathogenesis of the disease is unclear. This fluid is largely a sink containing a complex mixture of molecules, including a large concentration of hyaluronan, other proteoglycans, degradative enzymes, and serum proteins, many of which inhibit or degrade cytokine function. Furthermore, as the pathways and factors controlling the flux into the joint space are ill understood, we have not pursued analysis of cytokines in synovial fluid. In contrast, cytokine expression in synovial membrane is likely to be of greater relevance to the pathogenesis of RA, as this is the principal site of immune and inflammatory activity. The source of human RA synovial membrane is usually joint replacement surgery, which can provide large numbers of cells but has the limitation that this tissue is obtained only from late stages of the disease. On occasion, small, active samples from early stage disease are available from arthroscopic biopsies.

Since cytokines are local messenger molecules, we first investigated mRNA production as an index of synthesis. Due to the restricted sample size available and the large number of potentially relevant mediators to probe for, Northern blot hybridization and slot blotting techniques were developed for this purpose. With the capacity to strip and reprobe filters a number of times, it became possible to rapidly generate abundant data on cytokine expression in the rheumatoid synovium (19, 20). These techniques have now been replaced by more sensitive methods such as reverse transcriptase polymerase chain reaction (RT-PCR).

Pro-Inflammatory Cytokines

IL-1 and TNFα protein were readily detected in synovial fluid (18, 21–24). In the synovium, at the mRNA level these cytokines can be detected by blotting

(20) and by in situ hybridization (25, 26). Immunohistological localization of these protein products has demonstrated predominant expression in macrophages (25, 27). These proteins were also detected in the short term in in vitro cultures of the entire mixture of cells derived by enzymatic disaggregation of the synovial membrane (20, 28). Of importance was the observation that IL-1 and TNFα could be detected by bioassay of synovial membrane cultures, and hence they were present in quantities able to signal effectively.

Subsequently, as other proinflammatory cytokine and growth factor cDNAs were cloned, their mRNAs and proteins were also detected in RA synovial tissue. These cytokines included interleukin 6 (IL-6) (29–32), interferon α (IFNα) (24), granulocyte macrophage colony stimulating factor (GM-CSF) (33–35), macrophage colony stimulating factor (M-CSF) (36), and leukocyte inhibitory factor (LIF) (37–39). Many of these studies used osteoarthritis tissue or synovial fluid for comparison. Usually the same cytokines were produced, but at a lower level.

Currently we are unaware of any published data concerning the proinflammatory cytokine interleukin 12 (IL-12) (40) in rheumatoid joints or cultures. In our laboratory (FM Brennan, H Thomssen, P Green, RN Maini, M Feldmann, unpublished data), we have detected low levels of IL-12 in supernatants from RA joint cell cultures. The levels were within the biologically active range (20–200 pg/ml), with a mean of 79 pg/ml for p40 IL-12, and a mean of 30 pg/ml for p75 heterodimer. It is thus conceivable that IL-12 is important in the late stages of the disease process. In view of the predominance of $CD4^+$ Th1 cells in RA joints, with very few Th2 cells (see later), and that IL-12 is a powerful stimulus for skewing the Th1/Th2 ratio (41), it is likely that early in the disease process IL-12 may be importantly involved. In the latter stages IL-12 may also be involved in maintaining the Th1 preponderance and in driving cytokine production. Neutralizing experiments are needed to evaluate its potential role in the latter process. In collagen-induced arthritis, it has recently been reported (42) that IL-12 may replace the need for mycobacteria, markedly augmenting the incidence of arthritis in DBA/1 mice injected with Freund's incomplete adjuvant and collagen type II.

The cytokines identified as major contributors in the hyperplasia of rheumatoid synovial fibroblasts include platelet-derived growth factor (PDGF) (43–45), fibroblast growth factor (FGF), (45–47), and transforming growth factor β (TGFβ) (45, 47–50). Which of these is of major importance has not been evaluated experimentally, although immunohistological techniques have demonstrated co-expression of PDGF and FGF (45, 51) and of basic FGF with TGFβ (47) in RA synovial tissue. The presence of these and other cytokines in RA synovium is summarized in Table 1.

Table 1 Cytokine expression in RA Synovial Tissue

Cytokine		mRNA	Protein	References
Proinflammatory Cytokines				
IL-1α & β	(interleukin 1)	Yes	Yes	(18, 20, 23, 25, 26 28, 93, 103, 116)
TNFα	(tumour necrosis factor alpha)	Yes	Yes	(21–28)
LT	(lymphotoxin)	Yes	+/−	(22, 96)
GM-CSF	(granulocyte macrophage colony stimulating factor)	Yes	Yes	(33–35)
M-CSF	(macrophage colony stimulating factor)	Yes	Yes	(36)
IL-6	(interleukin 6)	Yes	Yes	(25, 29–32)
LIF	(leucocyte inhibitory factor)	Yes	Yes	(37, 39)
IL-II	(interleukin II)	?	?	
Onco M	(Oncostatin M)	?	?	
IL-2	(interleukin 2)	Yes	+/−	(19, 36)
IL-3	(interleukin 3)	No	No	(36)
IL-7	(interleukin 7)	?	?	
IL-7	(interleukin 9)	?	?	
IL-15	(interleukin 15)	Yes	Yes	(162)
IFNα	(interferon alpha)	Yes	Yes	(24)
IFNβ	(interferon beta)	?	?	
IFNγ	(interferon gamma)	Yes	+/−	(19, 25, 96)
IL-12	(interleukin 12)	Yes	Yes	(unpublished Feldmann M)
Immunoregulatory Cytokines				
IL-4	(interleukin 4)	?	No	(84)
IL-10	(interleukin 10)	Yes	Yes	(67, 85, 91, 92)
IL-13	(interleukin 13)	Yes	Yes	(unpublished Klareskog, L)
TGFβ	(transforming growth factor beta)	Yes	Yes	(48, 48, 50, 68, 68–71, 84)
Chemotactic Cytokines				
IL-8	(interleukin 8)	Yes	Yes	(53–55, 60, 61)
Gro α	(melanoma growth stimulating activity)	Yes	Yes	(60)
MIP-1α	(macrophage inflammatory protein 1 alpha)	Yes	Yes	(60, 63)
MIP-1β	(macrophage inflammatory protein 1 beta)	Yes	Yes	(60)
MCP-1	(monocyte chemoattractant protein 1)	Yes	Yes	(56–60)
ENA-78	(epithelial neutrophil activating peptide 78)	Yes	Yes	(62)
RANTES	(regulated upon activation T cell expressed & secreted)	Yes	Yes	(64)
Mitogenic Cytokines				
BDGF	(Vascular endophelial cell growth factor)	Yes	Yes	(184, 185)
FGF	(Fibroblast growth factor)	Yes	Yes	(44, 46, 47, 50, 51)
PDGF	(Platelet-derived growth factor)	Yes	Yes	(43, 45, 51)

Chemotactic Cytokines (Chemokines)

Many of the features of the rheumatoid synovial environment, such as the selective accumulation of VLA4$^+$ CD45RO$^+$ T cells and activated macrophages in the membrane, and of polymorphonuclear cells in joint fluids, suggest a possible role for leukocyte chemoattractant molecules such as chemokines. This superfamily of low MW peptides (7–15 kDa) has a conserved four-cysteine motif and consists of at least two subfamilies: the C-X-C (α) chemokines such as interleukin 8 (IL-8), melanoma growth stimulating activity (GRO α), and epithelial neutrophil activating peptide 78 (ENA 78), all of which predominantly attract neutrophils; and the C-C (β) chemokines such as RANTES (regulated upon activation normal T cell expressed and secreted), MCP-1 (monocyte chemoattractant protein 1), and MIP-1α (macrophage inflammatory protein 1α), which chiefly recruit T cells and monocytes (52).

Chemokines could be released by a number of cells present in RA joints, including endothelial cells, fibroblasts, macrophages, and lymphocytes. Members of both subclasses have been implicated in the pathogenesis of RA. Data from our laboratory and others have demonstrated the presence of IL-8 in RA synovial membrane cells (53–55); the production of other chemokines including GROα, MIP-1α, macrophage inflammatory protein 1β (MIP-1β) and MCP-1 (56–60) has subsequently been reported. In addition, immunohistochemical analyses of RA synovial tissue have demonstrated the presence of IL-8 (61), ENA-78 (62), MIP-1α (63), MCP-1 (57), and RANTES (64), predominantly associated with synovial tissue macrophages and, to a lesser extent, with endothelium and synovial tissue fibroblasts. Because the majority of the cells in the RA synovium are macrophages and T lymphocytes, β chemokines are likely to be important, although neutrophil chemoattractants such as IL-8, GROα, and ENA-78 may play a role in neutrophil accumulation within the synovial fluid.

Finally, in addition to their leukocyte chemotactic activity, chemokines may also be involved in other processes relevant to RA, such as connective tissue metabolism (for example, the α-chemokine connective tissue activation peptide CTAP III is elevated in rheumatoid plasma) and neovascularization (IL-8, GROα, ENA-78). However, it has not yet been ascertained which one(s) are the major contributors to leukocyte infiltration, or which will be therapeutically relevant. More recently, Kunkel and coworkers demonstrated expression of the β-chemokines MCP-1 and MIP-1α, and the α-chemokines ENA-78 and MIP-2 (the murine functional homolog of IL-8) (65) in murine collagen-induced arthritis (CIA). The earliest detectable levels of MIP-1α, MCP-1 and MIP-2 were observed 4 weeks after initial collagen challenge, a time-course paralleling that of disease development, whereas the time-course for ENA-78 was much slower

(8 weeks). Passive immunization of CIA mice with antibodies against either MIP-1α or MIP-2 resulted in both a delay in disease onset and a decrease in disease severity. Interestingly, anti-IL-10 treatment increased the expression of MIP-1α and MIP-2, associated with enhanced leukocyte infiltration in the joints, suggesting a homeostatic role for IL-10 in chemokine regulation (66) as well as its previously reported role (67) in regulating TNFα and IL-1β.

INHIBITORS OF IMMUNE ACTIVATION AND INFLAMMATION

Cytokines generally regarded as possessing immunoregulatory and inhibitory properties were discovered and subsequently cloned after the proinflammatory cytokines. These molecules include TGFβ, IL-4, IL-10, and IL-13. A number of studies from different groups have documented that TGFβ is abundant in both the precursor, inactive, form and the active form in rheumatoid joints (48, 68–71). However, whether TGFβ actually functions in RA as an anti-inflammatory cytokine has been questioned because it also has the potential to be proinflammatory (72). Thus, if injected locally into the joints of normal rats, TGFβ resulted in a rapid leukocyte infiltration with synovial hyperplasia leading to synovitis (73, 74), whereas if injected systemically into rodents susceptible to arthritis, it antagonized the development of polyarthritis (75, 76). Furthermore, in a recent publication, it was reported that anti-TGFβ antibody, injected locally into the joint of rats with arthritis, diminished the ongoing inflammation (77). These studies indicate the multipotential properties of TGFβ, and the differential effects if injected systemically or locally into the joint. Moreover, TGFβ clearly has other proinflammatory effects, such as acting as a chemotactic factor for monocytes (78). On the other hand TGFβ is likely to be a key cytokine involved in repair and fibrosis in the joints. For example, while inhibiting production of metalloproteinases such as collagenase (68) and inducing TIMP (16), TGFβ also stimulates the production of type I and type XI collagen (79). Thus, locally TGFβ may promote reparative processes in arthritic synovial connective tissue scarring and tissue repair by inhibiting cartilage and bone destruction. However, in chronic lesions, overproduction of TGFβ could contribute to the ongoing damage by recruiting inflammatory macrophages and fibroblasts with the potential for tissue destruction, and also, as shown recently (RA Fava, unpublished observation), by its ability to promote angiogenesis through induction of vascular endothelial cell growth factor (VEGF).

In common with TGFβ, IL-4 also displays some immunoregulatory effects such as inhibition of LPS-induced IL-1, TNFα, PGE2, and 92-kDa gelatinase

production in human monocytes (80–83). However, in contrast to TGFβ, IL-4 has not been found in rheumatoid synovial tissue cultures (84) nor in T cells cloned from RA synovial biopsies (85), although it has been detected in reactive arthritis (86). This and other evidence suggest that CD4$^+$ve Th2-derived cytokines are not abundant in RA joints, and that CD4$^+$ve Th1 cells predominate in this site (87). It is possible that the lack of IL-4–producing CD4$^+$ Th2 cells contributes to the pathogenesis of RA, and this has led to suggestions that IL-4 may be a useful therapeutic agent (88) (NA Mitchison, unpublished observation). This latter group demonstrated that the addition of recombinant IL-4 to RA synovial tissue organ cultures resulted in the inhibition of proinflammatory cytokine production (88). Using dissociated synovial cell cultures (89), we did not observe such a significant inhibition of proinflammatory cytokine production with IL-4; indeed, the expression of TNF-R on the surface of cells increased significantly, which could conceivably increase the responsiveness to TNFα. These effects aside, it is interesting to note that IL-4 production in RA joints is defective, and there is a report suggesting that the incidence of allergies is lower in RA patients (90).

IL-10 also has profound anti-inflammatory and immunoregulatory effects. Its presence has been documented in RA peripheral blood (91) and synovial joints by RT-PCR of fresh frozen biopsies, immunostaining of fresh frozen biopsies, and by assay of 24-h culture supernatants of dissociated joint cell cultures (67, 92). Furthermore, we found that in these RA synovial cell cultures, the endogenous IL-10 produced is functional, since inhibition of its activity using a neutralizing monoclonal antibody enhanced TNFα and IL-1 production (67). Conversely, addition of recombinant IL-10 to these cultures inhibited TNFα and IL-1 production by approximately 50%. In a similar study but using synovial tissue organ cultures (93), exogenous IL-10 also inhibited IL1β although IL-4 was more potent, and additionally IL-4 (but not IL-10) induced the production of the native inhibitor of IL-1, the IL-1 receptor antagonist, (IL-1ra). Although IL-10 is not a potent inducer of IL-1ra, we have shown recently that IL-10 (but not IL-4) induces the production of the endogenous TNF inhibitors, i.e. soluble TNF receptors from monocyte cultures, while also downregulating surface TNF receptor (TNF-R) expression (94). Thus, many of the properties of IL-10 are compatible with its being a major immunoregulator. However, not all of its properties are immunosuppressive, and its B cell stimulatory effects (95) may be important in the production of rheumatoid factors.

IL-13 also has inhibitory features resembling those of IL-4. It has not yet been quantitated in RA joints. However, preliminary data (L Klareskog, unpublished observation) suggest it can be detected by immunostaining in RA synovium.

ARE THERE DISCREPANCIES BETWEEN mRNA AND PROTEIN LEVELS FOR CYTOKINES IN RA JOINTS?

In contrast to the cytokines discussed in the previous section (IL-1, TNFα, IL-6, IL-8, GM-CSF), other cytokines detectable at the mRNA level were not abundant at the protein level but were present at low levels. These include cytokines derived principally from T cells, including IL-2, lymphotoxin (LT), and IFNγ (19, 36, 96). However, more recently, using more sensitive methods, T cell–derived cytokine proteins have been visualized by immunostaining; with this procedure, several groups have shown that IFNγ-producing T cells are not rare, with up to 0.5% of the total synovial T cells expressing IFNγ at any one time in joints of longstanding RA patients (85, 97) (L Klareskog, JS Smolen, unpublished observation). Other T cell cytokines, however, were not often detectable at either protein or mRNA level, e.g. IL-4 (84).

A variety of conclusions have been drawn on the basis of these observations. The extreme position has been taken by Firestein & Zvaifler (98), who have used these results as an argument that T cells do not have much role in the chronic, established stages of rheumatoid arthritis; instead, they argue, the process is perpetuated by cytokine interactions between other cells, including fibroblasts and macrophages. Alternative interpretations of the same data are possible, in view of the different physiology of T cells and macrophages. First, the low frequency of Th1-secreted products such as IFNγ and IL-2 may be sufficient to maintain T cell–dependent immune inflammation. Second, T cells migrate to their target tissue and deliver cytokines in a polarized manner, directly to their target cell. It would thus not be anticipated that large amounts of cytokines should be present in supernatants of T cells. Thirdly, since the evidence that T cells produce only low levels of cytokines, it has become apparent that cytokine inhibitors are abundant in body fluids. For IL-2, IFNγ, LT, etc, these are the soluble cytokine receptors, shed from the surface of cells. These effects would all contribute to limited detection of T cell cytokine. The major cause of limited T cell cytokine detection now appears to be that chronic inflammatory sites such as RA joints contain abundant quantities of cytokines capable of diminishing T cell cytokine synthesis. These include TGFβ (99), but probably the most important in RA is IL-10 (100, 101), since the addition of a neutralizing anti-IL-10 antibody to RA synovial membrane culture was able to upregulate IFNγ production in a proportion of cultures within 24 h (67).

CYTOKINE REGULATION

From the beginning of studies of cytokine expression in RA synovium, aspects of cytokine regulation there appeared different from what may have been

expected for in vitro activated cells. Most important was the consistent pattern of cytokine production, with all samples producing essentially the same pattern of cytokines. This was regardless of the duration of the disease or therapy, even with potential cytokine synthesis inhibitory drugs such as corticosteroids. In stimulated macrophages, IL-1β production greatly exceeds that of IL-1α at mRNA or protein level (102). In RA joints the production of IL-1α mRNA was relatively high, suggesting that IL-1 was either regulated differently or mostly was not coming from activated cells of the macrophage lineage (20).

The regular presence of cytokines in all rheumatoid joint synovial samples suggested that, unlike what is reported with normal cells stimulated in vitro, where cytokine expression is transient, cytokine expression in RA synovial tissue was likely to be prolonged or even continuous. This hypothesis was evaluated by placing RA synovial membrane cells in culture in the absence of extrinsic stimulation and ascertaining mRNA levels at various times. For example, in RA cultures, IL-1α mRNA persisted for the duration of the culture period (5–6 days) in the absence of extrinsic stimulation; in contrast in mitogen-stimulated peripheral mononuclear points cell cultures, mRNA expression was transient and shut off within 24 h (20).

The above results indicated that the signals regulating prolonged cytokine synthesis in rheumatoid joint cells were present in these RA synovial membrane cell cultures and hence could be analyzed. The problem at face value was quite daunting, with a heterogeneous mixture of cells producing a plethora of cytokine and noncytokine signals. Since Fell, Saklatvala and others (103–105) had shown that IL-1 (described as ‘catabolin’) was of importance in the initiation of destruction of cartilage and bone, we chose initially to analyze the regulation of IL-1 in rheumatoid synovial membrane cell cultures. While LPS is the usual experimental inducer of IL-1, possibly relevant in reactive arthritis, it is not likely to be important in RA: Instead we evaluated the effect of blocking cytokine inducers of IL-1. Since TNFα reportedly was a potent inducer of IL-1 (106), we used neutralizing antibodies to TNFα and lymphotoxin in RA synovial cultures to evaluate its role. The results were striking: within 3 days, IL-1 bioactivity had virtually disappeared. IL-1 mRNA levels were reduced much earlier. From this experiment, we concluded that TNFα was the major signal driving IL-1 synthesis in RA SM cultures (28).

This result prompted analysis of the production of other cytokines dependent on TNFα. Anti-TNFα antibodies also inhibited the production of another proinflammatory cytokine, GM-CSF (34). In addition to being a growth factor for monocytes in hematopoiesis, GM-CSF also activates mature monocytes and macrophages. It has been implicated in the pathogenesis of RA, based on the observation that it is produced spontaneously in RA synovialcell cultures.

GM-CSF induces and maintains HLA class II expression on RA synovial cells (35), in addition to regulating myelopoiesis (107). It may also affect other cell types in RA tissue as it can augment neutrophil-mediated cartilage degradation and adherence (108). In addition to GM-CSF, other cytokines including IL-6, IL-8 (109), and IL-10 (67) were all found to be induced by TNFα in these RA synovial cell cultures. The results obtained with pathological human tissue (synovium) are probably a reflection of the events that take place in a normal host defense response, as judged by the sequential appearance of TNFα, IL-1, and IL-6 in the serum of mice or monkeys injected with LPS or gram negative bacteria, and on the greatly reduced IL-1 and IL-6 production that follows neutralization of TNFα by an anti-TNFα antibody (110).

The role of TNFα in upregulating the production of other proinflammatory cytokines is not a completely unidirectional process. It has been reported that TNFα production can be upregulated by IL-1, GM-CSF, and IFNγ, and it is likely that such effects do take place in vivo. However the cytokine interactions are not symmetrical. Thus, blocking IL-1 in RA joint cell cultures using recombinant IL-1 receptor antagonist (IL-1ra) protein does not diminish TNFα production, but it downregulates IL-6 and IL-8 production (109). There is evidence that proinflammatory cytokines such as TNFα or IL-1 have an autocrine effect and can regulate their own synthesis. However, little evidence suggests that this is important in the RA joint: IL-1ra does not inhibit the production of IL-1α or IL-1β as judged by ELISA (109).

TNFα seems to be the cytokine at the apex of the proinflammatory cytokine cascade or network. So an important question is: What regulates TNFα production in rheumatoid joints? Currently this is unresolved. However, we have preliminary evidence that it is T cell–dependent, as T cell depletion in RA joint cell cultures diminishes TNFα production (FM Brennan, C Hawrylowicz, RN Maini, M Feldmann, unpublished observation). However, the critical T cell–derived signals to the monocytic cells, the major source of TNFα, are not yet known. Dayer and his colleagues (111, 112) have produced evidence that cell surface signals from T cells are involved in regulating IL-1 and probably TNFα in co-cultures of T cells and macrophages, with CD69 and CD11β playing an important role in this process.

CYTOKINE RECEPTORS

While it is useful to document the full range of cytokine expression in an inflammatory site, in isolation this information is not necessarily indicative of the extent or potential for cytokine signaling. The final outcome ultimately depends on two other important features, namely, the appropriate expression of cytokine receptors and the local concentration of cytokine inhibitors.

With the identification of cytokine receptors, the cDNA cloning and expression of many receptor chains, and the production of relevant antibodies, it has become possible to study cytokine receptor expression in small samples of diseased tissue. The technology used for receptor analysis has changed from measurement of mRNA expression, initially used for the IL-2Rα (or Tac) chain (113), to radioligand binding through to the current use of monoclonal antibodies. With the realization that most cytokine inhibitors are derived from the surface receptor by enzymatic receptor cleavage, it is evident now that mRNA analysis for cytokine receptors is not informative, as it does not discriminate between the cell surface signaling form and the inhibitory soluble forms.

TNF receptor expression has been studied most thoroughly. Both the p55 and p75 TNF surface receptors are upregulated in active RA tissues at both the protein and mRNA level (114). This is the case all over the synovium, including the areas abutting the sites of erosion (115) and in the endothelial cells. Of particular interest was the observation that macrophages at the cartilage pannus junction and the endothelial cells also produce TNFα (Figure 2), indicating the potential for autocrine stimulation. The expression of other cytokine receptors for IL-1 (116), GM-CSF (117), and IL-6 (unpublished) has been analyzed, but whether levels are normal or altered is not clear.

SOLUBLE CYTOKINE RECEPTORS

It is now well documented that the extracellular ligand-binding domain of most of the single transmembrane cytokine receptors is also found in biological fluids in a soluble form. Most of these soluble receptors are cleaved from the surface receptors of the cells by proteolytic enzymes, as in only a few instances is there evidence for a truncated mRNA, generated by alternative splicing (118, 119).

The first soluble receptor to be convincingly documented was that derived from the IL2Rα chain (113), but as this is of low affinity, its functional relevance as a cytokine inhibitor in vivo has not been clearly documented. TNF inhibitors were detected in urine and serum almost concurrently in 1988–1989, by the groups of Dayer (120), Olsson, (121) and Wallach (122), and they were subsequently characterized as the extracellular domains of the two TNF receptors. Using antibodies to these proteins, ELISA assays were developed, and soluble TNF-R (sTNF-R) were observed in normal serum at levels of 1–4 ng/ml, with the p55 lower than p75. In RA serum, levels of both were elevated, and even more so in the synovial fluid, where levels 3–4 times those of serum are reached. Furthermore, sTNF-R levels in plasma correlated with disease activity (123, 124). These results of upregulated TNF inhibitor in RA both systemically and locally are of interest, as they help exclude the possibility that a major contribution to the pathogenesis of RA is failure to produce inhibitory

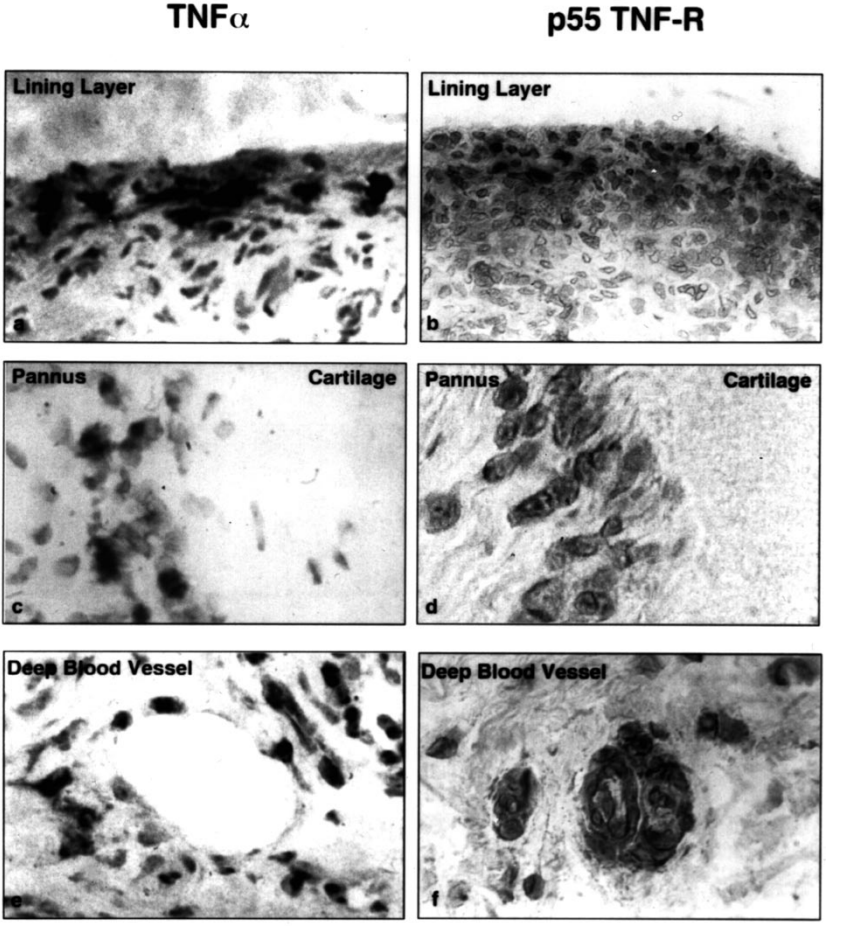

Figure 2 TNFα and p55 TNF-R expression in rheumatoid synovium. TNFα (*left panel*) and p55 TNF-R (*right panel*) colocalization was demonstrated by immunohistology in synovial lining layer (*a* and *b*) at the cartilage pannus junction (*c* and *d*) and in endothelial cells in deep blood vessels (*e* and *f*). Modified and reprinted by kind permission of *Arthritis Rheum.* (Chu et al, 1989, 34:1125–32 and Deleuran et al 1992, 35:1170–78).

factors normally. Indeed we observed that in RA synovial cultures sTNF-Rs were produced at concentrations capable of neutralizing a significant proportion of the TNFα generated (125). These results indicate that local production of cytokine inhibitors is capable of diminishing disease activity, and cytokine activity is partially downregulated by endogenous inhibitors.

Soluble IL-1 receptor has also been detected in RA tissues, initially in synovial fluid (126). This was first found as an IL-1β binding protein and was subsequently identified using monoclonal antibody as the type II IL-1R. This receptor is not involved in signaling and appears to function not only as a decoy on the cell surface, but also as an inhibitor (in its soluble form) as it binds proIL-1β (preventing its processing) and mature IL-1β, but not IL-1ra (127). Other soluble cytokine receptors have also been identified in RA. These include soluble IL-6 receptor (FM Brennan, A Cope, P Green, RN Maini, D Novick, M Feldmann, unpublished observation), which is not an antagonist but, in contrast, is an agonist (128, 129), and soluble IFNγ receptors (FM Brennan, A Cope, P Green, RN Maini, D Novick, M Feldmann, unpublished observation).

IL-1 Receptor Antagonist

The third member of the IL-1 family, the IL-1 receptor antagonist (IL-1ra) is the only known cytokine receptor antagonist (130). It has high affinity for membrane IL-1 receptor (type I and II), but due to the capacity of IL-1 to activate cells at very low receptor occupancy, a considerable molar excess ($\approx 100 : 1$) of IL-1ra is needed to inhibit IL-1. The expression of IL-1ra has been analyzed in RA joints; thus mRNA levels are upregulated, as is production of the protein in RA synovial fluid and joint cell cultures (131–134), which is immunolocalized to CD68 positive macrophages within the synovium (116, 132). Of key importance is the ratio of IL-1ra to IL-1, which in a recent report (135) from RA synovial cultures ranged from 1.2 to 3.6, well below the 100-fold excess of IL-1ra needed to neutralize IL-1 bioactivity. Thus, it is not surprising that bioactive IL-1 is found in the majority of RA culture supernatants (28). Normal joint tissues express very little IL-1ra, so IL-1ra production is upregulated in the disease process but not sufficiently to neutralize IL-1. It is of interest that a study of the ratio of IL-1ra:IL-1 in the synovial fluid of patients with Lyme arthritis indicated that the patients with the most favorable outcome had the highest ratio (136).

CYTOKINE ANALYSIS IN ANIMAL MODELS

While there is no animal model of arthritis that entirely mimics RA, a variety of models have been generated that resemble RA in many important respects. These models are being analyzed to provide possible insights into the

pathogenesis of RA and to help develop therapeutic strategies. There is very little published data concerning cytokine expression in the synovium in animal models. Most of the available data concerning the role of cytokines in animal models have emerged from administering cytokines into the joints of mice or from therapeutic studies in these mice. More recently the development of transgenic mouse models of arthritis has provided an alternative way in which to analyze the role of cytokines in arthritis.

Originally a synovitis with proteoglycan degradation was seen to occur if IL-1 alone, or in combination with TNFα, was injected into the joint of a rabbit (137, 138). Subsequently, injection of IL-1 (139, 140) or TNFα (141, 142) into collagen-immunized mice or rats accelerated the onset and increased the severity of arthritis. Therapeutic studies in mice indicated the involvement of these proinflammatory cytokines in the normal course of arthritis. For example, several groups have shown that collagen-induced arthritis (CIA) in mice may be treated effectively with anti-TNF antibody or other TNFα inhibitors. Thus, monoclonal antibodies to TNF ameliorated CIA when administered prior to disease onset (143, 144). In addition, anti-TNF monoclonal antibody treatment was used successfully after disease onset and was found to reduce inflammation, as judged by footpad swelling, as well as joint destruction, as judged by histological analysis of the joints (145). Analogous results have been obtained using IgG-TNF-receptor fusion protein as an inhibitor of TNFα activity (144, 146, 147). The fusion proteins used and the monoclonal antibody (TN3) are likely to have blocked murine lymphotoxin as well as TNFα. The effects of blocking IL-1 have also been studied in CIA using several approaches. Wooley reported using daily injections of IL-1 receptor antagonist, which resulted in a delay in onset and a reduced incidence of arthritis (148). More recently (149) the combination of neutralizing rabbit anti-mouse IL-1α and IL-1β administered during the pre- and post-arthritic periods was reported to reduce the incidence and severity of CIA. We have also demonstrated amelioration of established CIA using a neutralizing antibody to the type I IL-1 receptor (R Williams, LJ Mason, RN Maini, M Feldmann, unpublished observation).

More recently, we have started to analyze the kinetics of cytokine expression during the early stages of DBA/1 CIA using immunolocalization studies. A consistent finding to emerge from these unpublished studies is that TNFα is expressed in the lining layer of the synovial membrane at the time of arthritis onset; IL-1 is also detected but at a slightly later stage of the disease (1–2 days after the onset of clinical arthritis). Thus, these results of cytokine/anticytokine treatment in vivo are consistent with the analysis of human RA tissues in vitro, and they can be extended to the whole animal with its greater complexity, including cell recruitment.

Other results have been paradoxical, such as those with TGFβ and IFNγ. As noted previously, the injection of TGFβ into footpads of normal or collagen-injected rats induced an arthritis or accelerated its onset (73, 74), while systemic administration of TGFβ to collagen-injected mice ameliorated CIA (75, 76, 143). These studies indicate the multipotential properties of TGFβ (72) and its differential effects when injected systemically or locally into the joint.

IFNγ is a cytokine commonly believed important in the pathogenesis of autoimmune diseases, a theory first proposed in a hypothesis concerning the mechanism of autoimmunity, in which IFNγ was envisaged as necessary for upregulation of antigen presentation (150). Evidence to support this concept appeared rapidly in human studies (151–153). Transgenic mice overexpressing IFNγ in various tissue sites were found by Sarvetnick and her colleagues to develop autoimmunity in the pancreas, retina, and at the neuromuscular junction (154, 155). Localized overexpression of a single cytokine yielding autoimmunity is not reproduced by other proinflammatory cytokines, e.g. TNFα or IL-1 in diabetes models. The importance of IFNγ as a local mediator of inflammation in RA has also been confirmed in CIA. Thus, local injection of IFNγ into the footpads of collagen type II–immunized mice accelerates the onset of arthritis and increases the severity of disease (156). Conversely, systemic administration of IFNγ ameliorates CIA, and treatment with anti-IFNγ may exacerbate or ameliorate the disease depending on the mAb used and the timing of treatment (157, 158). The reasons for this are not clear, but IFNγ has a multitude of effects; some such as downregulation of B cell activity or the antagonism of IL-1–induced bone resorption (159) may be beneficial. It will be interesting to evaluate whether IFNγ or IFNγ receptor knockout mice, backcrossed onto DBA/1 mice, will be able to develop CIA.

The most studied transgenic model of arthritis is in mice expressing a modified human TNFα transgene, with the AU-rich 3′ untranslated region replaced by that of β globin (160). These mice develop an erosive arthritis that can be prevented with anti-human TNFα antibody. The arthritis is the major pathology in this mouse, and it is not yet understood why the joints are so sensitive to TNFα, as there is disregulated expression of TNFα in all tissues. The arthritis does not appear to be lymphocyte dependent, because arthritis develops in TNFα transgenic mice backcrossed to RAG knockouts, which do not possess functional lymphocytes (D. Kioussis, personal communication). The spontaneous appearance of arthritis in multiple strains of human TNFα transgenic mice provides strong supporting evidence for the pathogenic relevance of TNFα in driving the arthritis process in vivo.

We have studied cytokine expression in the joints of these huTNFα transgenic mice by culturing synovial cells obtainedfrom inflamed joints. The technique

chosen was dissociation of synovial tissue cells and subsequent culture in the absence of extrinsic stimulation, mimicking the work with human synovium. Abundant human TNFα in a bioactive form was detected, but paradoxically very little or no murine TNFα was seen, suggesting that autocrine TNFα stimulation was not important. However, upon stimulation with LPS, such synovial cell cultures will produce murine TNF. IL-6 was also abundant, as was IL-10, but only a little IL-1 was detected. As human TNFα reportedly binds the murine p55 TNF-R and not the p75 TNF-R, these findings suggest that signaling through the p55 TNF-R is in itself sufficient to produce autocrine and paracrine cytokine induction and to initiate the disease process (D Butler, A-M Malfait, RN Maini, M Feldmann, FM Brennan, unpublished observation).

In human RA synovial cultures, TNFα is the major stimulus for IL-1 production (28). In the mice transgenic for human TNFα, therapy with a neutralizing antibody to the IL-1 receptor (type I) is protective (161), as is the anti-TNFα antibody as originally described (160). This suggests that much of the activity of TNFα in this in vivo model is mediated by its capacity to generate IL-1 or to synergize with it.

ROLE OF CYTOKINES IN DIFFERENT ASPECTS OF ARTHRITIS

Role of Cytokines in T Cell Function in RA

There are abundant T cells in a RA joint, averaging 20–30% of the mononuclear cells in the synovium. Most are $CD4^+$ $CD45RO^+$. There is little evidence for local proliferation, so that continuous recruitment is important. Recruitment involves upregulation of adhesion molecules, found in synovial endothelium, and expression of chemokines, also found there. The cell surface phenotype of the T cells is consistent with chronic activation, with $> 50\%$ expressing DR, VLA-1, VLA-4, VLA-7, but $< 10\%$ usually expressing IL-2R. T cells do not survive in the absence of stimulatory signals from the T cell receptor or cytokines. Cytokines present in the joint that may be important in sustaining T cell survival and function include IL-2 (low amounts), IL-7, and IL-15. The latter is relatively abundant in RA joints (162). IL-10 reportedly prevents apoptosis in B lymphocytes and T lymphocytes (163, 164), and as it is abundant in RA joints it may have a role in sustaining the survival of T cells there, although its inhibitory effects are also expressed (see previously).

T Cells in Rheumatoid Joints Are Enriched in Th1-Like Cells

A number of T cell cloning studies have been performed in RA, although none is very detailed. In all of them, the production of IL-4 was not a common event,

so that the majority of the cells were Th1-like, producing IL-2 and IFNγ (87, 165). In the mouse, IL-10 is produced by Th2 cells (166), but in RA joints, T cells produce large quantities of IL-10 (85), consistent with previous studies with human T cells in which IL-10 was found in both Th1 and Th2 subsets (167). The preponderance of Th1 cells in the joint would be in keeping with the presence of the major inducer of Th1 cells IL-12 being detectable in small but bioactive quantities (unpublished data).

Which Cytokines Are Responsible for the Damage to Cartilage and Bone?

In rheumatoid arthritis, the destruction of cartilage and bone occurs by erosion mainly at the junction of cartilage, bone, and synovium, a region known as the *pannus* (14). This destruction progressively invades the bone and spreads over the cartilage, occurring in two forms, one highly cellular, expressing essentially the same mixture of cytokines as in the active synovium and associated with active erosion. Another subset is relatively acellular and expresses chiefly TGFβ; it appears to be a site of repair rather than destruction (168). Normal cartilage cells can produce a wide spectrum of cytokines (168), including IL-1, TNFα, IL-6, and can respond to these also. Hence it is not clear which cytokines are important in cartilage damage, although IL-1 and TNFα are clearly implicated as they induce destructive matrix metalloproteinases, such as MMP-1 (collagenase) and MMP-3 (stromelysin), that are involved in cartilage destruction (104, 169, 170), and they also induce bone resorption in in vitro cultures (105, 171). There is increasing evidence that IL-6 may inhibit bone formation and induce bone resorption through its stimulatory effects in osteoclasts (172). Thus it is interesting that IL-6 gene knockout mice do not develop erosions of bone (173). In view of the fact that IL-6 signals via gp130 (128), a receptor chain shared with other cytokines including LIF, IL-11, and oncostatin M, it is conceivable that they may also be involved in bone destruction in RA. IL-11 is a considerably more potent inhibitor of bone nodule formation than is IL-6 (174), whereas in contrast, there is evidence to suggest that LIF may stimulate bone formation (175). Little is currently known about the effect of oncostatin M on bone metabolism, or indeed whether IL-11 or LIF induce bone resorption.

Are the Pathogenesis of Synovitis and Joint Destruction Equally Dependent on the Same Cytokine Network?

A key question is whether the destruction of connective tissue, the major source of long-term joint problems in RA, is driven by the same cell interactions and cytokines as are implicated in the synovitis. This question has been raised by several workers in the field (176, 177); for example, Bresnihan noted that

conventional therapy while improving synovitis has less effect on bone erosion according to the X-ray progression.

Therapeutic trials of anticytokine and other new therapies have the potential to resolve this issue, by demonstrating that effective therapy resolves the synovitis and protects the joints from destruction. All the anti-TNFα clinical trials so far are too short term to assess for joint protection radiographically, but the reduction in pro MMP-3 production reported suggests that joint protection may occur with long-term reduction of TNFα (178).

Neovascularization in RA

The rheumatoid synovium is a much more cellular mass of tissue than the normal synovium and is characterized by an abundance of blood vessels, thus increasing the delivery of cells and molecules to areas of inflammation (179). The neovascularization that takes place in RA is central for the maintenance of the disease process, since inhibition of angiogenesis using either the microtubule stabilizer Taxol or AGM-1470, a synthetic angiogenesis inhibitor, blocks arthritis in a rat model of CIA (180, 181).

The process of neovascularization in RA involves angiogenic cytokines such as VEGF, an endothelial cell-specific mitogen that promotes the growth of new blood vessels (182) and renders the vasculature hyperpermeable in vivo (183). VEGF has been detected in RA joint tissue and synovial fluids by two independent groups (those of Fava and of Koch) and was associated with strong expression of VEGF mRNA by synovial tissue macrophages and of VEGF receptors by microvascular endothelial cells (184, 185). Hypoxia is a strong inducer of VEGF release and VEGF receptor expression (186, 187); the RA joint reportedly is hypoxic due to pressure on vessels during movement (188), although other inflammatory stimuli within the RA joint may also contribute to VEGF production from macrophages. In this respect, it is interesting to note that TGFβ has recently been found to induce VEGF release from RA synovial cells (R Fava, unpublished observation). VEGF also is chemotactic for endothelial cells, which may perpetuate angiogenesis.

Finally, it has been reported that a large number of cytokines elevated in RA, including TNFα, TGFβ, FGF, as well as chemokines such as IL-8 (189), and possibly other C-X-C chemokines such as GRO α and ENA-78 (S Kunkel, unpublished observation) may also exhibit angiogenic activities.

The Cytokine Network in Other Forms of Arthritis

Compared to rheumatoid arthritis, little is known of the cytokine network in other forms of arthritis. Due to the availability of operative samples at joint replacement, osteoarthritic (OA) synovium has been used as a control for many of the studies in RA. It is not, however, a good comparison, because the diseases

differ in their underlying etiology: RA has an autoimmune component, while OA does not. By the stage of joint replacement, it is evident that an extensive phase of tissue destruction has occurred in OA, with much of this attributable to an inflammatory component. This is perhaps why the cytokine pattern appears to be relatively similar to that in RA. There is no clear-cut distinction, as the cytokines TNFα, IL-1, and IL-6 are all expressed, albeit at lower average levels (28, 29). Chemokine expression has also been noted, including that of IL-8 (53) and MCP-1 (57). Anti-inflammatory cytokines such as IL-10 (67) and TGFβ (69) are abundantly expressed in OA, as in RA. Perhaps one of the few differences is in the bioactivity of TNFα in synovial cultures. While all RA synovial cultures produce bioactive TNFα, a significant percentage of OA synovial cultures do not. This is due to excess free sTNF-R (125). For most of its long history, OA has had a tendency to induce excessive bone formation (e.g. osteophytes), which must involve a cytokine pattern different to that in RA. What prompts the change to a bone destructive form of OA would be interesting to understand, due to its therapeutic implications. Other forms of arthritis such as psoriatic arthritis and infection-related reactive arthritis have not been well documented at the cytokine level.

The arthritis in reactive arthritis, in contrast to RA, is not destructive, and as such synovial tissue is much harder to obtain. One interesting comparison has been performed at the mRNA level by Simon et al (86). Reactive arthritis specimens often produced IL-4 mRNA (6/8), but only 2 out of 10 of those from the rheumatoid did, even if analyzed by RT-PCR. This admittedly limited study suggests that the Th1/Th2 balance may be critical for a self-limiting arthritis, and the excess of Th1 cells in rheumatoid arthritis may be important in its persistence (NA Mitchison, unpublished).

LESSONS FROM CLINICAL RESEARCH AND TRIALS

Cytokine Antagonists as Therapeutic Agents

In the previous sections we have analyzed the experimental evidence for the evolving concept that the broad spectrum of cytokines locally produced in joints provides a molecular basis for the observed pathology. Now we examine the extent to which it has proven possible to validate the role of each cytokine in vivo by using specific antagonists of high affinity and specificity such as monoclonal antibodies, soluble cytokine inhibitors, and in the case of IL-1, IL-1ra, in the therapeutic setting. The accumulating evidence suggests that TNFα is not only an inflammatory mediator in its own right but also is the key regulator of the production of other cytokines implicated in rheumatoid inflammation such as IL-1 (28), GM-CSF(34), IL-6 and IL-8 (109). We have therefore espoused

TNFα as a prime target for therapeutic trials. However, antagonists of IL-1 and IL-6 have also been tested in the clinic by others; in the case of IL-1 antagonists, the results of randomized clinical trials have not yet been published in full, and anti-IL-6 therapy has thus far been tested only in an open-label trial.

Anti-TNF Clinical Trials

The availability of a chimeric (mouse $\times$ human) monoclonal anti-TNFα antibody cA2 (from Centocor, Malvern, Pennsylvania), with an affinity of 1.8 $\times$ 10^{-9} kDa and with neutralizing activity on a variety of biological activities of TNFα in vitro (190), provided an opportunity for testing hypotheses for a new treatment of RA (191, 192). Chimerization of the antibody (75% human immunoglobulin) should reduce immunogenicity and increase the half-life of murine antibodies (193). This monoclonal antibody (cA2) had been shown to be nontoxic in preclinical safety tests on monkeys and human volunteers in the dose range that was predicted to be therapeutic, based on the dosage needed to ameliorate collagen-induced arthritis in DBA/l mice (145).

Accordingly in 1992/1993, in an open-label trial, we administered cA2 by two to four intravenous infusions over two weeks, in a total dose of 20 mg/kg body weight, to 20 patients who had chronic erosive RA unresponsive to multiple disease modifying drugs (DMARDs) such as gold, methotrexate, and salazopyrine (194). To avoid the confounding carry-over effects of previously administered DMARDs and of simultaneous ongoing anti-inflammatory therapy, patients ceased DMARDs for a minimum of four weeks and were stabilized on a fixed dose of nonsteroidal anti-inflammatory drugs (NSAIDs) and/or corticosteroids in this period. The treatment with anti-TNFα monoclonal antibody led to rapid improvement in every patient in all indices of disease activity used for monitoring patients. Such modalities included clinical evaluations such as the number of swollen and tender joints, the degree of pain experienced, the duration of stiffness of joints on waking in the morning, locomotor function, the physician's assessment of overall benefit, and the patient's assessment of improvement. Laboratory measurements of inflammatory activity such as the erythrocyte sedimentation rate (ESR) and C-reactive proteins (CRP) also showed marked reduction. The improvement from baseline reached its maximum within the first four weeks, with mean values of change exceeding 60–70%, and lasted 8–22 weeks (median 12 weeks). Since the trial was primarily to test the safety of the therapy, particular attention was paid to vital signs and side effects during and following therapy. The infusions were tolerated without any change in cardiorespiratory function or body temperature, and patients seemed to be free of adverse events during the trial period.

The magnitude of clinical response and reduction in CRP was convincingly reproduced in a randomized, double-blind, multicenter, placebo-controlled trial

on 73 patients in 1993/1994 in four European centers (195). In this trial, entry requirements were active erosive rheumatoid disease and a history of failure to respond to at least one DMARD. Having withdrawn from DMARDs, patients were stabilized on a fixed dose of anti-inflammatory drugs for four weeks. Thereafter, a single intravenous infusion of cA2 at two doses (1 mg/kg or 10 mg/kg) or placebo infusion (0.1% human serum albumin in normal saline) was administered over 2 hr to 24 patients in each group (25 in the 1 mg/kg group because of premature dropout of one patient in the other). An impressive change was seen in all measurements and is illustrated in Figure 3 for the number of swollen joints and CRP. The primary endpoint of this trial was the achievement of improvement in a composite index of disease activity defined by Paulus et al (196), which discriminates between DMARDs and placebo effects in randomized controlled trials. According to this index, a positive result was defined as significant improvement in four out of six variable measurements defined as: 1. at least 20% improvement in continuous variables (tender and swollen joint scores, duration of morning stiffness, and ESR); 2. at least two grades of improvement in patient's and observer's assessment of disease activity.

When the Paulus criteria were applied in the trial, a striking benefit was observed in the majority of patients treated with anti-TNFα when compared with those treated with a placebo (Figure 4). According to the predetermined primary endpoint of the study at four weeks, 79% of patients significantly improved, compared with 44% at the lower dose. In contrast, 8% of patients receiving placebo achieved the same level of response at 4 weeks. In order to define

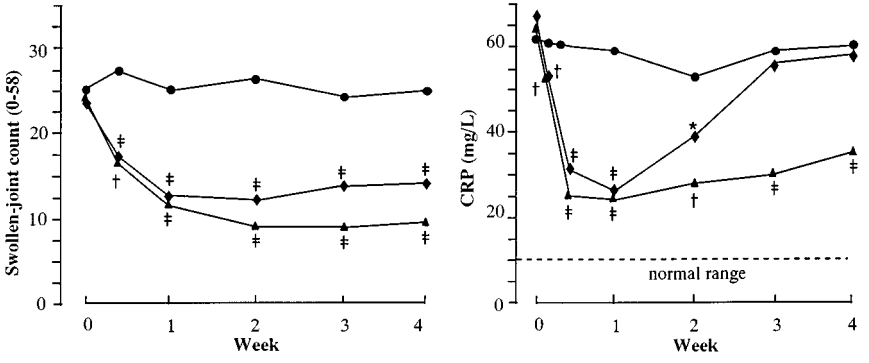

Figure 3 Swollen joint count assessment and C reactive protein measurement in patients receiving placebo (● or cA2 anti-TNF mab at 1 mg/kg (♦), and 10 mg/kg (▲). Values are means of 24 patients at each point (25 for 1 mg/kg group). Significance versus placebo: $*p < 0.05$, $†p < 0.01$, $††p < 0.001$. Reprinted by kind permission of *The Lancet* (Elliott et al, 1994, 344: 1105–10).

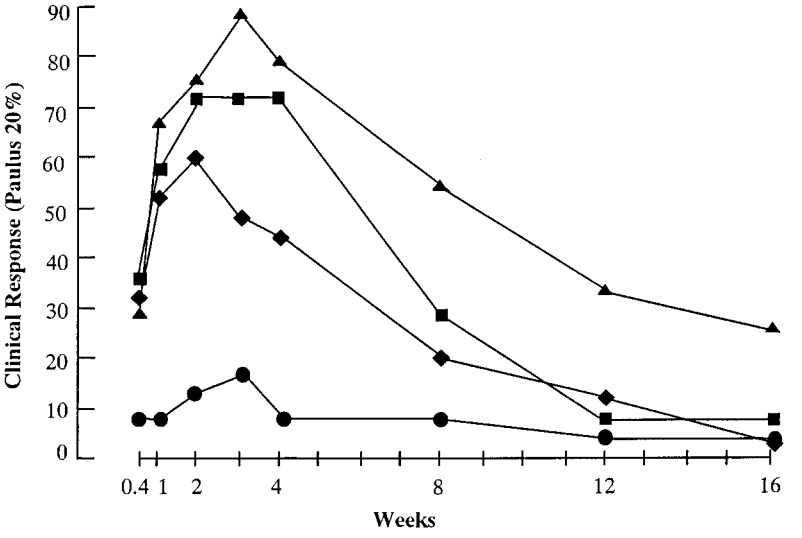

Figure 4 Duration of clinical response assessed by the Paulus disease activity index following placebo, 0.1% HSA, $n = 24$ (●); and cA2, anti-TNF mab at 1 mg/kg, $n = 25$ (♦); 3 mg/kg, $n = 14$ (■); and 10 mg/kg, $n = 24$ (▲). Based on data in (Elliott et al, 1994, 344: 1125–27).

the proportion of patients who showed an even more marked improvement, the Paulus Criteria were arbitrarily reset by increasing the threshold of response to 50% improvement in continuous variables, and the data were reexamined. Using these rigorous criteria, 58% of patients benefited at the high dose of anti-TNFα monoclonal antibody and 28% at the low dose (195). This profile of data at four weeks seemed outstanding when compared with the effect of other drugs tested in randomized placebo-controlled trials.

Analysis of the dose-response relationships of parameters of disease activity in patients revealed that in the initial two weeks, the proportion of responders at the low dose (1 mg/kg) matched that at high dose (10 mg/kg). The magnitude of response in the individual clinical parameters of disease activity (such as number of swollen and tender joints, ESR, and CRP), generally showed a 60–70% change from baseline. The difference in response between the low and high doses lay in the duration of the response.

The duration of response was further examined by following all treated patients to relapse of disease. Upon relapse, a further infusion of anti-TNF was given. According to protocol, 14 patients who had initially received placebo then subsequently received an intermediate 3 mg/kg dose of anti-TNFα. This permitted an evaluation of the duration of response at three doses of anti-TNF.

The median duration of response was clearly dose-dependent (Figure 4), lasting 3 to 8 weeks in this dose range and persisting for over 16 weeks in 25% of patients receiving the high dose of anti-TNF antibody (197).

The pharmacokinetics of the antibody have given insight into the duration of response. Preliminary analysis shows that the Cmax following single infusions of the 1 mg/kg and 10 mg/kg of cA2 were 28 mg/L and 328 mg/L, respectively. The kinetics appear to fit a two-compartment model. Using the mean serum concentrations over time following a 10 mg/kg dose, calculations show that the half-life of the antibody is around 18 h (at one week). More significantly, in interpreting clinical responses, detectable levels persist for 10–12 weeks at the high doses. At lower doses the antibody persists for a correspondingly shorter duration (197). It is of interest that at 1–5 mg/ml of cA2, TNFα effects in rheumatoid joint cell cultures are inhibited (109). Levels above 1–5 mg/ml are maintained depending on the dose administered. It explains why the initial magnitude of the response and proportion of patients is the same for the three doses used, but diverges with the passage of time and a fall in circulatory level below the therapeutic threshold.

Since the efficacy of a single treatment cycle has a finite duration of several weeks and because the disease relapses in all cases, the question arises whether the anti-TNFα monoclonal antibody therapy is efficacious in controlling chronic disease if given in repeated infusions. Only limited data are available currently, although an ongoing study on 105 patients receiving repeated cycles will attempt to address this point. However, what is known from the experience gained in retreating seven patients with up to four cycles of anti-TNFα monoclonal antibody following relapse is that it was possible to use the antibody repeatedly and to demonstrate an equivalent magnitude of control of disease with subsequent infusions, although there was a trend toward a shortening of effect in some patients (198). The results encourage the view that TNFα blockade is likely to be beneficial as long-term therapy. There is as yet no evidence for the emergence of a TNFα-independent pathway, which could have been the case in view of the potential redundancy of the cytokine network with numerous proinflammatory mediators.

Since TNFα is known to be of importance in host resistance to infections, we have taken an interest in documenting infective events in treated patients. However, RA patients are liable to infections to a greater extent than the healthy control populations (199) and may be influenced by a reporting bias under continuing close scrutiny. The only accurate way of assessing an increased incidence is to compare the type and rate of infections against a population of RA patients stabilized on equivalent background therapy, but not receiving antiTNFα monoclonal antibody. This has been possible in the randomized

controlled study in the first four weeks, and results showed an incidence of six episodes in 49 patients, or 12% (three upper respiratory, two skin infections, and one episode of pneumonia in anti-TNFα treated patients) versus one upper respiratory tract infection in 24 patients (4%) receiving placebo therapy. The significance of these predominantly minor infections will require further analysis following a more extensive experience, but the result does not suggest that it is a limitation of therapy. The observation of a trend toward normalization of indices of previously depressed cellular immune functions common in RA patients (200–202) following anti-TNFα therapy, such as lymphocyte proliferation in vitro (203) and delayed hypersensitivity reaction to recall antigens in vivo (204), is reassuring in this context.

An unexpected side effect in these clinical trials was the development of IgM class anti-ds-DNA antibodies some weeks following cA2 treatment in 7 out of 91 patients (6%) without evidence of clinical SLE. Three of these seven received another infusion, and in only one did the titer of antibody rise; in one it remained unaltered; and a third showed reversal to normality. In all patients the antibody levels spontaneously resolved over several months. The pathogenesis of anti-ds-DNA antibodies in a minority of RA patients is unexplained at this point, but it is noteworthy that other anti-rheumatic drugs can induce ds-DNA autoantibodies (205). The development of lupus serology following TNF depletion is consistent with observations made by Jacob and McDevitt in NZB/W mouse model of SLE, in which TNF deficiency was associated with a genetic polymorphism of the TNF locus in this mouse (206). More recently, in anti-IL-10–treated NZB/W F_1 mice (207), remission was attributed to induction of TNF, since anti-TNF therapy caused rapid recurrence of severe disease. It would appear that in a subpopulation of RA patients, anti-TNFα may similarly predispose to anti-ds-DNA antibodies, and there is a risk that a proportion of these patients might develop a lupus-like syndrome with long-term therapy. These studies suggest that the cytokine network is fundamentally different in RA and SLE (208)

Trials of TNFα blockade with other biological agents have been reported by other groups. In a double-blind dose-ranging study, a humanized mouse monoclonal antibody (CDP 571, Celltech, Slough, UK) was administered to 24 patients in a single dose at 0.1, 1, and 10 mg/kg (209). In this antibody the hypervariable regions were grafted into a human IgG_4 immunoglobulin. The results when compared with 12 placebo-treated patients showed that it was well tolerated, and significant improvement appeared in a composite disease-activity score and in some of the individual disease-activity criteria. Unlike with cA2, however, no significant difference was noted in the swollen joint score. Reductions in CRP were also observed, thus confirming the results with

cA2, but the comparative dose-response relationships suggest that the potency of CDP571 was less than that of cA2. Whether this reflects differences in epitope specificity, affinity, or isotype (IgG_4 versus IgG_1) is unknown, but further investigation could illuminate the mode of action of the two anti-TNFα antibodies. CDP571 also induced anti-ds DNA antibody production (D. Isenberg, personal communication, European Rheumatology Meeting, 1995).

Engineered soluble TNF receptors linked to Fc portion of IgG, which are divalent and act as potent inhibitors of TNF (210), are being developed for therapy of disease by the pharmaceutical companies. Such soluble TNF receptor fusion proteins should be less immunogenic than monoclonal antibodies partly derived from mice, and they may be therapeutically active, since they bind TNFα with high affinity. The bioengineered immunoglobulin-like molecule might also be expected to increase its circulating half-life. Two such products are undergoing therapeutic trials in RA patients. A p75 soluble-TNF-R, linked to Fc of IgG_1 (Immunex), or placebo, has been administered in a Phase I study to 16 patients with refractory RA at the University of Alabama at Birmingham (211, 212). Three patients in each group received an intravenous loading lose of 4, 8, 16, and 32 mg/m^2, followed by subcutaneous injections twice weekly for four weeks, of 2, 4, 8, and 16 mg/m^2 in the four groups, respectively. Additionally, four patients received placebo injections. No serious adverse events were recorded. Clinical responses did not show a dose-response improvement, but at day 31 following commencement, there was a 40–55% mean improvement in painful, swollen, and tender joints in anti-TNFα-treated groups compared with 22 to 25% improvement in the placebo group; however, these differences were not statistically significant. CRP was reduced by 25% compared with 13% in the placebo group, and this also was not statistically significant. Roche has used a p55 soluble TNF-receptor IgG_1 fusion protein in ongoing trials but has not disclosed data, though abstracts and meeting presentations claim efficacy.

We may conclude from these trials with several different agents that TNFα antagonists are likely to be beneficial in RA, but the precise indications for their use and their limitations as a generic therapy or the relative efficacy of individual biological variants must await further development. The current data are short term, and assessment of long-term safety will need further clinical trials.

Mechanism of Action of Anti-TNFα Antibody Therapy

Rheumatoid inflammation results from the conjoint effects of multiple mediators locally produced by many cell types. We have presented evidence based on clinical trials of the marked dose-dependent anti-inflammatory effects of anti-TNFα therapy. However, it remains to be established which of the many biological effects of TNFα (213) is of central importance in amelioration of symptoms and reduction of acute phase proteins. Our current hypothesis suggests two

main mechanisms of action: first, local deactivation of the proinflammatory cytokine cascade following neutralization of excess amounts of TNFα at the inflammatory site; and second, an interruption of cellular recruitment and traffic into rheumatoid joints as a result of a reduction of TNFα-dependent expression of vascular adhesion molecules, and of chemokines.

The remarkable reduction in levels of acute phase proteins (e.g. CRP) following within 72 h of infusion of anti-TNFα antibody cA2 (194) indirectly points to an effect on the cytokine network. The production of CRP by the hepatocyte is known to be regulated by cytokines, chiefly by IL-6, but also by other cytokines such as LIF and IL-11, signaling via the gp130 receptor. We have measured serum IL-6 levels before and after infusion of anti-TNFα antibody, and they have shown a rapid decline that parallels a fall in the CRP concentration in the blood (194, 197, 214). Predictably, the subsequent rise in CRP is also closely associated with an increase in IL-6 levels, occurring earlier at the lower dose (1 mg/kg) when compared with the higher dose (10 mg/kg) of anti-TNF. A reasonable explanation of this sequence of events is that neutralization of TNFα in the synovial compartment interferes with the cytokine network (see earlier), thereby reducing IL-6 production as occurs in rheumatoid joint cell cultures (109). Cytokine deactivation is mirrored by a fall of IL-6 levels in blood and is followed by a reduced end-organ response, as judged by a fall in CRP synthesis by the hepatocyte.

Cytokine blockade and diminution of other downstream inflammatory pathways is probably of major importance in the rapidity of reduction in pain, stiffness, swelling, and tenderness of joints. The best objective evidence of an anti-inflammatory effect currently available is based on histopathological examination of serial biopsies of synovial tissue obtained from multiple sites of knee joints before infusion with anti-TNFα antibody and 14 days later. Examination of coded multiple sections of synovium by two blinded observers overcame observer bias and variability of the cellular response in different regions of the joint, and results clearly showed a reduction in the cellularity of synovium (197, 215). Reduction is seen in the number of cells/high power field in the synovial lining layer, infiltrating mononuclear cells (predominantly macrophage-like cells), and perivascular lymphoid aggregates.

The reduced cellularity of synovium also supports our second hypothesis, suggesting a reduction in trafficking of blood-borne cells into diseased joints. A simultaneous significant increase in the number of circulating lymphocytes accompanying a fall in synovial infiltrate is in keeping with reduced influx into joints. That this is dependent on TNFα-modulated expression of adhesion molecules is supported by the decreased expression of ICAM-1, VCAM-1, and E-selectin in the biopsies (197, 215, 216). ICAM-1 and VCAM-1 are

detected at vascular endothelial sites in the synovium as well as in many of the lining layer cells. E-selectin was detected on up to 28% of vascular endothelial cells of capillaries and venules. Following anti-TNFα therapy, we observed a significant reduction in E-selectin expression in endothelium and also an overall reduction in VCAM-1 and ICAM-1 expression in the lining layer.

Recently, the detection of soluble forms of the adhesion molecules ICAM-1, VCAM-1, and E-selectin in blood has become possible by ELISA. These molecules are derived from proteolytic cleavage of the surface form (217), and their release correlates with increased surface expression following activation by TNFα and IL-1 on cultured endothelium (218, 219). Elevated serum levels of ICAM-1, VCAM-1, and E-selectin have been observed in RA, and soluble VCAM-1 and ICAM-1 appear to correlate with disease activity (220, 221).

In our European multicenter, randomized placebo-controlled study with anti-TNF antibody, we showed a dose-related reduction in soluble E-selectin and soluble ICAM-1 levels (221), but interestingly not of VCAM-1 levels. The reduction paralleled parameters of clinical response, ultimately returning to pretreatment levels closely in parallel with recurrence of symptoms of inflammation. It was noteworthy that in cA2-treated patients (1 mg/kg and 10 mg/kg pooled), there was very good correlation between clinical response and reduction in serum E-selectin (221). Recruitment of cells into the synovial tissue involves activation of cell surface integrins such as LFA-1 and ICAM-1, and chemokines are powerful signals for upregulating the affinity of these integrin molecules. Interestingly, anti-TNFα in vitro diminished IL-8 production (109), and preliminary evidence indicates that this also occurs in vivo. It is thus very likely that the diminished recruitment of cells into joints partly explains the prolonged duration of clinical benefit.

In conclusion, the reproducible clinical benefit of TNFα blockade in RA using antibodies has clearly underlined the importance of understanding the role of cytokines in arthritis. It may not be farfetched to consider that treating earlier in the diseases process, or with longer term TNFα blockade, it may be possible to redress the homeostatic balance and obtain longer term benefit (remissions) and possibly cures in some patients.

IL-1 and IL-6 Antagonists

Recombinant human interleukin-1 receptor antagonist (IL-1ra), manufactured by Synergen, is a candidate drug for use in RA since it successfully blocks the effects of IL-1 (130). The requirement for 90% receptor occupancy to achieve IL-1 blockade means that the agent has to be used repeatedly at relatively high dose levels as the molar ratio of IL-1ra to IL-1 needs to be >10. In a randomized double-blind controlled trial, 175 patients with RA were divided into nine groups that received 7, 20, or 200 mg, once daily, three times a week,

or once weekly for three weeks. Subsequently all patients received a dose once a week for four weeks. Of these patients 14% withdrew due to lack of efficacy or adverse events. Data in abstract form (222) shows that at three weeks patients receiving daily injections showed significant improvement compared with patients receiving weekly injections, but no dose-response relationship was apparent in patients receiving the three-dose levels of daily injections. Significant but relatively modest reduction in CRP ($< 40\%$) was observed. The effects apparently lasted three to seven weeks in some patients.

Recombinant type I soluble IL-1 receptor (sIL-R, from Immunex), which inhibits the binding of IL-1 to its cell-associated receptor, has been used in a Phase I randomized single center study utilizing intra-articular injections in the knee. Four doses were evaluated in four RA patients in each group (total 16) and compared with a placebo injection in four patients. Following 25, 100, 250, and 500 mg injection into a knee, a dose-related reduction in knee circumference was observed at 48 h and 7 days, reaching significance only at the dose of 250 mg (223).

In another randomized, double-blind study reported in abstract form, sIL-1R was administered by daily subcutaneous injection for 28 days. Twenty-three patients with RA were treated with 125, 250, 500, and 1000 mg/m^2 daily and compared with patients receiving a placebo. No patient met predetermined criteria for significant improvement (224). In this study the choice of a type I soluble IL-1 receptor (rather than type II) may not have been optimal, since it binds IL-1α with greater affinity than does the predominant IL-1β form secreted in RA joints. It also preferentially binds endogenously produced IL-1ra in joints, which thus may negate its potential benefit, and possibly increases the bioavailability of IL-1β by displacement from sIL-R (127). The limited clinical trial data available on IL-1 blockade, by the agents described above, do not permit any definitive conclusion concerning the relative merits of blockade of IL-1 and TNFα. Other IL-1 blocking agents or different clinical protocols may yield greater benefit, but at the moment it is not proven that IL-1 blockade is effective in RA; this is in contrast to TNFα blockade by monoclonal antibodies, in particular cA2 (194, 195, 209).

A murine anti-IL-6 monoclonal antibody has been administered in an open-label study to eight patients with refractory RA (225, 226). Improvement in pain and number of tender joints was observed, lasting two months. An impressive fall in CRP and ESR occurred in this period. The authors report an increase over baseline in circulating levels of immunoreactive IL-6 following treatment. The significance of this trial and IL-6 data is unclear, but it merits further investigation.

CONCLUDING REMARKS

In this chapter we have attempted to encapsulate the impact of cytokine research in providing fresh insight into molecular mechanisms of disease. These developments have been dependent in equal part on the availability of a novel array of biological agents and their application, as specific antagonists or agonists, in experimental model systems and clinical trials. Interim conclusions can be drawn and give rise to cautious optimism on the potential of these experimental approaches in unravelling complex biological responses and assessing the therapeutic value of neutralizing or augmenting specific cytokines in the clinical situation.

Among the encouraging results is the demonstration that monoclonal antibodies and other biological products that act as specific cytokine antagonists can be administered to patients safely, at least in the short term. Despite the pleiotropy intrinsic to the action of many cytokines, it appears that their blockade can lead to impressive clinical benefits—for example, following anti-TNF therapy. Moreover, repeated administration leads to reproducible effects, arguing against redundancy in the biological systems involved in disease. However, complete and sustained remission of disease has not been achieved, and it is unclear whether current modes of long-term delivery of biologicals will be tolerated without evoking allergic responses in a proportion of patients or inducing immune responses, which by neutralizing their biological activity will lead to loss of efficacy of the therapeutic principle. It is also not yet known whether long-term neutralization or augmentation of the activity of a cytokine may be associated with unanticipated side effects. More experience is clearly necessary, although the speed of progress is such that availability of biologicals as licensed drugs in the treatment of arthritis in two to three years is a distinct possibility. In the meantime, improved systems of delivery of biologicals, for example, by gene therapy, may provide exciting prospects for development.

Among the challenging questions to be addressed in the near future is the possibility that better results may be obtained in controlling RA by combination therapy. Thus, more than one cytokine might be targeted simultaneously, or proinflammatory cytokine blockade may be combined with anti-T cell therapy. An equally compelling case can be made for testing biologicals in combination with existing disease-modifying drugs of proven efficacy, such as methotrexate and salazopyrin. Whichever approach is used, the high frequency of placebo responders in RA makes the testing of drugs in randomized controlled trials mandatory. The equally pressing need to use validated criteria of benefit in clinical trials is being addressed by co-operation at international level. These efforts should simplify the task ahead and allow comparisons to be made.

The definition of therapeutic targets by the use of biologicals has stimulated the reevaluation of mechanisms of action of established drugs, and it has also stimulated the search for chemical drugs that carry the specificity of biologicals. The race between the approaches has begun, and the potential strengths and weaknesses of each one are all too apparent. So are the enormous opportunities to improve significantly on the limited benefit of drugs in primary and secondary prevention of disabling arthritic disorders.

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SOMATIC HYPERMUTATION OF IMMUNOGLOBULIN GENES

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ABSTRACT

The relationship between somatic hypermutation and affinity maturation in the mouse is delineated. Recent work on the anatomical and cellular site of this process is surveyed. The molecular characteristics of somatic hypermutation are described in terms of the region mutated and the distinctive patterns of nucleotide changes that are observed. The results of experiments utilizing transgenic mice to find out the minimum *cis*-acting sequences required to recruit hypermutation are summarized. The hypothesis that V gene sequences have evolved in order to target mutation to certain sites but not others is discussed. The use that different species make of somatic hypermutation to generate either the primary or secondary B cell repertoire is considered. Possible molecular mechanisms for the hypermutation process and future goals of research are outlined.

INTRODUCTION

To find out the structural basis for the great functional diversity of antibodies (1) was an early focus of research in immunology. The answer was gradually revealed. The elucidation of the four chain structure (2) led to the question of how individual heavy and light chain molecules could vary so as to be able to recognize different antigens and yet maintain the same basic structure. Sequencing of monoclonal immunoglobulins obtained from patients with myeloma (3, 4, 5) showed that the amino-terminal domains of the polypeptide chains were heterogeneous, whereas the carboxy-terminal domains fell into a restricted number of classes. How could the genetic information for heavy and light chain molecules

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be encoded so as to produce such a structure? Was every antibody sequence represented in the germline or were there a limited number of germline elements that underwent somatic diversification?

Somatic hypermutation, which is the alteration of a germline immunoglobulin sequence by introduction of nucleotide changes during the lifetime of a B cell, was originally postulated to provide an answer to such questions and was conceived to be a mechanism that could contribute, in part or in entirety, to antibody diversity (6, 7).

The major outcome of the initial characterization of the immunoglobulin gene loci by molecular cloning was that the somatic recombination of different germline-encoded gene segments constituted a principal source of immunoglobulin diversity (8, 9). The first indications that antibodies contained differences from each other came from sequencing of ten λ light chains from mouse myeloma proteins (10). This revealed that six were the same (the putative germline sequence), whereas the others bore one or two differences. Cloning of the mouse $V_\lambda 1$ germline gene (11) allowed the definitive demonstration that these $V_\lambda 1$ sequences from myelomas contained differences from the germline and that somatic hypermutation had occurred. Other studies analyzing immunoglobulins produced by various mouse myelomas and plasmacytomas (12–15) reached similar conclusions.

Much of the early evidence that somatic hypermutation accompanied the affinity maturation of antibodies came from studies in the mouse, and this is the species on which most of this review is focused.

SOMATIC HYPERMUTATION AND AFFINITY MATURATION

During the course of an immune response, the affinity of serum immunglobulin for antigen typically increases (16–18). Early work showed that structural alterations occurred in the antibodies produced during an immune response (19), but their exact nature was not known.

Somatic hypermutation plays a central role in antibody affinity maturation and is in large part responsible for the production of the secondary repertoire (20–30). This secondary repertoire is constituted by cells, which show improved antigen binding characteristics, and which are themselves descended from B lymphocytes triggered by the initial antigen challenge. The nature of these changes has been systematically investigated by immunizing with several different model haptens, e.g. *p*-azophenylarsonate (23, 24), 2-phenyl-5-oxazolone (25–28), phosphorylcholine (29, 30), and 4-hydroxy-3-nitrophenylacetyl (31, 32).

Sequencing of the V genes from hybridomas producing antigen-specific antibody revealed two types of changes during the immune response. First, the immunoglobulin genes were usually expressed in mutated form with the degree of mutation increasing during the course of the response. For example, following immunization with 2-phenyl-5-oxazolone (27, 28), there were essentially no mutations at day 7 (following primary immunization), but the extent of mutation was such that by day 14 no identical sequences were found. Second, the repertoire of germline V genes used in the response shifted. Thus, at day 7 and to a lesser extent at day 14, a V_HOx1/V_κOx1 gene combination predominated. Following secondary immunization, V_H/V_L gene combinations other than V_HOx1/V_κOx1 were found with greater frequency. This phenomenon, designated a repertoire shift, has also been found in other systems (31–33).

Improvements in antibody–antigen binding affinity can be brought about by alterations to the kinetic parameters of binding, namely on-rate and off-rate. In the 2-phenyl-5-oxazolone system, improvements to the V_HOx1/V_κOx1 pair are frequently achieved by decreasing the off-rate. By contrast, the repertoire shift away from V_HOx1/V_κOx1 is characterized by moving to antibodies that exhibit a high on-rate. It is these latter antibodies and their derivatives that dominate the later stages of the response (34), although the combination of V_κOx1 with alternative V_H genes is also found. It may be that the deep antigen binding pocket in V_HOx1/V_κOx1 limits the effective on-rate and that antibodies with a different geometry would not be so hampered and could give rise to further improvements. However, many secondary response antibodies use V_κOx1 with different V_H genes, e.g. members of the MOPC21 or J558 gene families (28). Generalization as to what germline genes will constitute a potential high affinity antibody is, therefore, not possible because on-going mutation may alter, i.e. either improve or worsen, the binding.

The course of the response to 2-phenyl-5-oxazolone is accompanied by a 100-fold increase in the antibody affinity for the antigen (28). Despite the influence of repertoire shift mentioned above, a striking correlation emerges between stage of immunization (primary, secondary, or tertiary), degree of somatic hypermutation and increase in affinity. Direct proof that somatic hypermutation alone can be responsible for alterations in binding affinity has come from several different lines of evidence. V_κOx1 light chains bearing single mutations were allowed to combine with the unmutated V_HOx1 heavy chains (35), and affinities were measured. The substitution of His34 by glutamine or asparagine increases affinity by about tenfold. In an alternative approach, decrease in affinity for antigen was directly attributed to somatic hypermutation (36). Hybridomas producing an antibody of specific idiotype (normally with a high affinity for p-azophenylarsonate) were established, and those that had barely detectable

affinity for this antigen were selected. Extensive somatic hypermutation of the canonical sequence implies that this process is responsible for the abolition of antigen binding. A third line of evidence was derived from mutagenesis experiments. The germline gene V_H186.2 encodes most antibodies against the hapten 4-hydroxy-3-nitrophenyl acetyl in C57BL/6 mice. A point mutation that causes Trp33 to be changed to leucine occurs in most high affinity antibodies. This nucleotide substitution was introduced into a germline gene, and the affinity was thereby raised tenfold (37). Conversely when an antibody bearing this mutation as well as many others was mutagenized in order to restore the wild-type sequence, the affinity dropped.

CELLULAR ASPECTS

Germinal centers are located in the secondary lymphoid follicles in the peripheral lymph nodes of human and mouse. Their involvement in somatic hypermutation was suspected from the temporal association between their development and the maturation of the immune response.

About 4 days following immunization, B lymphocytes migrate to primary lymphoid follicles, where they proliferate. Germinal centers then develop during the ensuing 3–6 days (38). Histologically, germinal centers can be divided into a basal dark zone of proliferating centroblasts and an apical light zone containing centrocytes (the progeny of centroblasts) as well as a network of follicular dendritic cells able to hold antigen on their surface (39, 40). By 3 to 4 weeks, centroblasts and centrocytes can no longer be found.

Proof that mutation occurs in germinal centers has come from immunohistology (38), sequencing of antibody genes from B cells within and without germinal centers (41), and sequencing of antibody genes from individual germinal centers (42). The mutating population of human B cells has been further characterized by sorting of tonsillar cells for expression of various surface markers and then sequencing antibody genes within these populations (43). From this study, it has been inferred that hypermutation occurs in surface IgD^-, $CD23^-$ centroblasts, but not in their precursor cells which express these two markers.

Peripheral blood contains B cells of differing origins and maturity. Both newly produced lymphocytes that have just been selected from the bone marrow into the peripheral blood and memory B cells will be present. Analysis of human peripheral blood B cells fractionated according to surface expression of IgM and IgD (44) shows, as expected, that naive IgM^+IgD^+ cells express V_κ genes with virtually no somatic hypermutation, whereas memory cells (IgM^-IgD^-) bear highly mutated genes. The effect of the age of the animal on the degree of somatic hypermutation has been investigated in two systems. In mouse Peyer's patches, the proportion of lymphocytes that bear mutations increases gradually

from birth and peaks at 5 months of age (45). In the case of humans, mutated V gene sequences have been recovered from the tonsils of a four-year-old child (46).

The germinal center is the site of both B cell proliferation and selection. The interplay between these processes must be regulated to generate efficiently B cells that produce high affinity antibodies. It has been inferred from mathematical modeling that cycling between distinct phases of proliferation and selection is the best way to achieve this outcome (47). The rationale is that a large pool of cells is produced during the proliferative phase which will increase the likelihood that an advantageous mutation will be produced and subsequently selected. If this theory is correct, then roles can speculatively be assigned to the populations of B cells found in actual germinal centers (48). It may be that centroblasts in the basal dark zone represent the proliferating population in which there is ongoing somatic hypermutation and that selection occurs following the interaction between centrocytes and follicular dendritic cells in the apical light zone. A corollary to this view of events within a single germinal center is that B cells can go through multiple cycles of proliferation and selection. One would predict that the evolution of highly mutated clones from those bearing fewer mutations could then be discerned. This has been substantiated (49–51) by analysis of B cells picked individually from germinal centers.

THE MUTATIONS

The Mutation Domain

Extensive analysis of somatic mutations in antibodies has revealed that mutations are largely confined to the variable domains and are rarely found in the constant domains (51–55). While they are detected throughout the variable domain, it is mutations in the complementarity-determining regions (CDRs) (28, 56) that have most frequently been implicated in improved antigen binding—a finding consistent with the fact that it is the CDRs that play the major role in direct antigen contact.

Mutations largely occur over a region of one to two kilobases, around the rearranged V-J gene segments (52, 57). They do not usually extend into the C-region, although rare mutations in the mouse Cλ1 gene have been described (55). On this point it may be relevant that, at the mouse λ loci, the C genes are quite close to the J clusters.

The mutation domain extends from a 5′ site within the leader intron (58–61), although sequences upstream of the transcriptional start site do mutate at a lower frequency (57–59), and continues through the V gene well into the J-C intron (62). Mutations occur in the framework regions as well as the hypervariable

regions of the rearranged V gene segments (14). Support for the concept that the process is targeted to a specific region of DNA came from the finding that nonfunctional V genes also bear mutations (63). Although most nucleotides in or close to the V segment can mutate, the mutations tend to be found in the complementarity determining regions (58, 59, and see below) rather than being randomly spread out.

Nature of the Mutations

The nature of the mutations observed in antigen-selected B cells will be determined by (i) the mutations that were originally generated in the germinal center, together with (ii) selection by antigen for those B cells carrying mutations that confer a selective advantage. The effect of skewing by antigenic selection can be considerable. For example, most day 14 hybridomas producing 2-phenyl-5-oxazolone-specific antibodies and that use the V_HOx1/V_κOx1 combination carry mutations in the His34 codon of V_κOx1 (27). A similarly dominant mutation of Trp33 to leucine is found in the V_H genes of B cells selected in response to 4-hydroxy-3-nitrophenylacetyl (31). These mutations, which characteristically are both in complementarity determining regions, are rarely found when these V genes are selected by other antigens (62, 64, 65).

How can the intrinsic specificity of the mutational mechanism (i.e. removed from the skewing effects of antigen selection) be studied? There have been studies of mutations in V-region flanking sequences (58–62) where antigenic selection does not operate, and the presence of hotspots that do not cause amino acid replacements has been documented (28). More recently, the fact that the immunoglobulin transgenes can act as substrates for hypermutation (66) has been exploited to analyze the intrinsic features of the hypermutation mechanism. Information has been gleaned from experiments in which either the transgene does not contribute to the expressed antibody of the cell [and is acting as a silent passenger target for mutation (67)] or in which the transgene is contributing to the antibodies directed against a wide variety of antigens and in which there is, therefore, no dominant skewing by a single antigen (64, 65). Here we focus on those aspects of mutation that appear intrinsic as they may give clues to the molecular mechanism responsible for the mutational process.

Nucleotide Substitution Preferences

The mutations introduced during hypermutation are predominantly single nucleotide substitutions (rather than insertions or deletions) (14, 15, 52), although they show a slightly increased tendency to occur in clusters (68). Transitions are commoner than transversions and, furthermore, as assessed on the coding strand, G bases are mutated more often than C bases and A's more than T's. This implies that the hypermutation machinery can discriminate between the

two DNA strands—i.e. there is strand polarity (67). This pattern of base substitutions is not restricted to transgenes but has also been found in human V_H genes from splenic B cells (69)—a population selected by many antigens so that the effects of selection by individual antigens can be ignored. However, heterologous genes, i.e. nonimmunoglobulin, behave in a more complicated fashion (70). A bacterial *neo* gene showed the characteristic polarity observed for immunoglobulin genes, but bacterial *gpt* and human β globin genes did not.

Hotspots

Mutations are not targeted randomly along the length of the V gene. Some residues are frequently targeted (hotspots), others rarely (coldspots). Hotspots can be defined, at least in part, by local DNA sequence. A consensus sequence Pu-G-Py- (where Pu = [A or G] and Py = [C or T]) has been proposed (71) as a favored target for mutation. Indeed, three of the major intrinsic hotspots that have been identified in the V_κOx1 gene are all located in serine codons (Ser26, 31 and 77) that are encoded by AGPy triplets and conform to this consensus. The Pu-G-Py-$\frac{A}{T}$ consensus can of course be translated in different reading frames, and both glutamine and alanine codons have been found to be hotspots when they occur in the context of this sequence. More recently, analysis of intrinsic hotspots in transgenes that carry nonimmunoglobulin sequences in place of the V segment (70) has lent further support to the proposal that the Pu-G-Py-$\frac{A}{T}$ consensus sequences are favored targets for mutation. However, not all Pu-G-Py-$\frac{A}{T}$ sequences are mutational hotspots, and this consensus is clearly insufficient to define a mutational hotspot. Local DNA features such as palindromes or inverted repeats may well play an important role (28, 72, 73).

GERMLINE V GENE SEQUENCES HAVE EVOLVED TO ENCOURAGE TARGETING OF SOMATIC MUTATION

Given that the hypermutation mechanism is intrinsically nonrandom and given also that it is likely to be more useful to target mutations during antibody affinity maturation to some parts of the antibody molecule (e.g. CDRs) rather than others (e.g. frameworks), it might be that V gene sequences have evolved so that hotspots are strategically located. This appears to be the case. The amino acid serine is unusual in that it is encoded by two types of triplet AGPy (that is, AGC and AGT) and TCN (that is, TCA, TCC, TCG, and TCT). The sequence AGPy conforms to the hotspot consensus; TCN does not. This provides a test of whether codon usage has evolved so as to favor local targeting of hypermutation. Combining these pieces of information suggests the hypothesis that one way of ensuring that serine codons in CDRs are more frequent targets for mutation than serine codons in frameworks would be to favor AGPy triplets for CDR

serines and TCN triplets for framework serines. This is indeed the case (74). Significantly, in the case of T cell receptor V genes, no equivalent skewed usage of serine codons is observed (74). (Although mutation may occur at low frequency in T cell receptor Vβ (but not Vα) genes (75), there is no evidence of somatic hypermutation playing any role in their functional diversification.) Thus, it appears that the codon usage of immunoglobulin genes has been selected during the course of evolution so that the most mutable residues are in positions where alterations will be most likely to lead to improved affinity.

Interestingly, there are conserved AGPy serines codons in framework 3 of both V_H and V_κ genes. The significance of this is uncertain, but given that experimental alterations to sites outside the CDRs can alter antibody affinity (76, 77), an intriguing possibility is that the rare conserved AGPy triplets in the framework regions have similarly been selected for their mutability. It appears that biased serine codon usage may be a general feature of antibody molecule design, because such a usage is also found in *Xenopus* V_H genes (78).

DNA SEQUENCES REQUIRED FOR RECRUITING HYPERMUTATION

Somatic hypermutation is restricted to the region around the rearranged V gene. Mutation is rarely observed in unrearranged V genes (14, 79), and only a low frequency of mutations has been observed in incompletely rearranged heavy chain loci bearing DH-JH integrations (80). Most of what is known about the sequences necessary to recruit hypermutation to the immunoglobulin V gene comes from studies of modified immunoglobulin transgenes.

Enhancer Elements

Most work has been carried out on the mouse κ light chain locus. Immunoglobulin κ transgenes mutate at a low rate (66). This suggested that transgenic animals would provide a useful approach to finding the minimal *cis*-acting sequences necessary for full hypermutation. Certain immunoglobulin κ transgenes hypermutate in a way similar to endogenous κ genes as regards both the type and the frequency of mutation (67, 81, 82). Both the transcription enhancer elements in the κ locus (the 3′-enhancer and the intron-enhancer/matrix attachment region) are necessary for effective hypermutation. The intron-enhancer/matrix attachment region may be the more important of the two because its removal abolishes detectable mutation, whereas constructs lacking the 3′-enhancer continue to mutate although at greatly reduced frequency (64). The other transcriptional regulatory element in the locus, the V_κ promoter, does not appear to contain specific sequences necessary for recruiting hypermutation. Transgenes carrying a β-globin promoter in place of the V_κ promoter continued to mutate (64).

However, such studies do not exclude the possibility that an active promoter is required for hypermutation.

Less is known about the regulatory elements in the heavy chain and λ light chain loci. Although mutation is readily observed in V_H (29, 31, 69) and Vλ (62) genes encoded by the endogenous loci, little success has been obtained so far in obtaining hypermutation from constructs bearing rearranged IgH or λ genes (83–85). Presumably, this reflects an absence of necessary *cis*-acting DNA sequences from the constructs that have so far been tested, although the presence of inhibitory sequences obviously cannot be excluded. In the case of one of the rearranged IgH transgenes (84), mutation of the transgenic V_H segment was detected in hybridomas in which *trans*-switching appeared to have recombined the transgenic V_H into the endogenous IgH locus. This observation supports the idea that the transgene may lack sequences located toward the 3′-end of the IgH locus necessary for proper hypermutation. In contrast to the failure with rearranged IgH transgenes, mutation (albeit probably at low frequency) has been obtained using transgenic IgH mini-loci in which the transgenic V_H segments undergo productive rearrangement to yield an antibody repertoire (86, 87). The reason why limited success is obtained here in contrast to failure with the rearranged IgH genes is unresolved.

The V Domain

Although the V gene is of course the target of hypermutation, several experiments have been conducted to ascertain whether it is required for recruiting hypermutation or whether the hypermutation mechanism can target heterologous sequences in place of the V (88–90). Recent experiments (70) have clearly revealed that the bulk of the V segment is not necessary for recruiting hypermutation. Different constructs lacking the leader, the leader-V intron, or the majority of the V gene segment up to the J segment were all effective as targets for hypermutation. Furthermore, heterologous sequences used to replace the V (a segment of the human β-globin gene or bacterial *neo* or *gpt* genes) all acted as substrates hypermutation. Indeed, the pattern of mutation (i.e. a predominance of nucleotide substitutions and a preference for transitions) in these heterologous targets was similar to that obtained with a V gene although polarity was less marked (70). These heterologous sequences also showed the characteristic nucleotide hotspots (conforming to the Pu-G-Py-$\frac{A}{T}$ consensus). Thus, although the V domain has evolved to act as a hypermutation substrate (see above), it does not actually contain sequences necessary for the recruitment of hypermutation.

In conclusion, the only elements in the immunoglobulin loci known to be required for recruiting hypermutation are in the region of the transcription enhancers. No evidence exists, however, that these elements are sufficient; and

the identification of the minimum DNA essential for recruiting hypermutation to a heterologous gene awaits definition.

PHYLOGENETIC ASPECTS

In human and mouse, somatic hypermutation seems to be implicated only in the production of the secondary, affinity-matured, antibody repertoire. However, this does not imply that hypermutation arrived late in evolution. Indeed, it contributes to antibody diversification in other mammals, e.g. in sheep (91), probably in rabbits (92), and in frogs (78, 93). There are also strong indications of its importance in even more primitive organisms such as members of the shark family (94).

The involvement of hypermutation in the generation of antibody repertoires in chicken is not fully resolved; the analysis is complicated by the extensive use of gene conversion in this species (97, 98). [Although the possible involvement of gene conversion in antibody diversification in human and mouse has been discussed (97, 98), there is evidence that it does not play a significant role (64, 99). For example, the variability seen in the germline V_κOx family is quite distinct from that seen in mutated V_κOx1 genes (64). An exception, however, is the case of a transgene, designed to favor gene conversion, which contains two closely linked V genes (100).]

The role of somatic hypermutation may have diverged in different species. In human and mouse, hypermutation is involved in the formation of the secondary antibody repertoire, whereas in sheep it appears to play a major role in the formation of the primary repertoire (91). This process occurs in the ileal Peyer's patches, and the extent of accumulated mutations is not reduced by attempts to exclude external antigen (91). Thus, somatic hypermutation may not always be intimately linked to antigen selection. Indeed, in primitive vertebrates such as *Xenopus* (93) and the cartilaginous fish (94, 101), hypermutation appears to occur, but the selection of B cells producing high affinity antibodies seems less developed than in mouse. It is noteworthy that germinal centers have not yet been identified in these species.

Hypermutation may first have arisen to diversify the primary immunoglobulin repertoire when only limited combinatorial diversity was available. For example, a member of the shark family, the little skate (*Raja erinacea*), contains light chain genes that are already joined (i.e. V-J integrated) in the germline. There is obviously no combinatorial or junctional diversity in this system, and yet hypermutation may well occur (102). Further support for the antiquity of the hypermutation mechanism is provided by the novel (non-immunoglobulin and non-T cell receptor) antigen receptor genes found in the nurse shark, which undergo extensive somatic hypermutation (103).

It has been noted (104), in a comparison of the protein coding sequences of human and mouse, that (similar to the hotspots associated with somatic hypermutation—see Germline V Gene Sequences Have Evolved To Encourage Targeting of Somatic Mutation), AGPy codons are more mutable than TCNs. This suggests that there are features common to evolutionary and somatic hypermutation. One can synthesize the following argument: Somatic hypermutation was initially adapted from a basic cellular mechanism in order to diversify the primary antibody repertoire, which was restricted by lack of combinatorial diversity (105). In such a situation, a strong selective pressure existed for immunoglobulin V gene sequences to evolve in order to ensure that mutation was preferentially targeted to regions whose alteration would be likely to lead to useful diversification. In some more recently evolved species, the hypermutation mechanism has a minor or no role in primary diversification but is more important in affinity maturation, where it occurs in cooperation with a specialized cellular selection machinery.

MECHANISMS FOR SOMATIC HYPERMUTATION

Little is known of the mechanism of somatic hypermutation. The features that must be accounted for in any model of the process are: (*a*) its occurrence during only a short period of B cell development; (*b*) targeting to the rearranged V gene; (*c*) dependence on both the 3′ and intron enhancers; (*d*) the mutation domain starting downstream of the promoter and extending well into the J-C intron; (*e*) base substitution preferences, and (*f*) strand polarity.

Various mechanisms for somatic hypermutation have been proposed, for example, gene conversion (97, 98), replication (106) and lag strand DNA synthesis (107). We focus on a further proposal that envisages a role for error-prone DNA synthesis (7) in somatic hypermutation. Indeed, several of the DNA polymerases involved in DNA repair lack the proofreading 3′-to-5′ exonuclease activity and therefore manifest a higher degree of misincorporation. Clearly, any model that involves the introduction of mutations not only requires errors/changes to be made to the DNA, but, if these errors are made on only one strand (as, for example, in DNA synthesis or repair), then the mismatches generated must not be repaired back to the original i.e. germline, sequence.

The requirement for the immunoglobulin κ enhancers for full hypermutation draws attention to a possible linkage between hypermutation and transcription. A further association between transcription and a specific form of DNA repair, i.e. nucleotide excision repair, suggests a model for somatic hypermutation involving the transcription factor complex, TFIIH, whose normal function is the recruitment of RNA polymerase II (108, 109). If, for example, sequences in the immunoglobulin locus (e.g. in the intron enhancer/matrix attachment region)

initiate local or regional single strand nicking, one can envision the TFIIH/ RNA polymerase II complex detecting these nicks on the template strand and recruiting an error-prone DNA polymerase. Such linkage with transcription could explain not only the requirement for the enhancer elements, but also the strand polarity and the fact that the hypermutation domain starts downstream of the promoter.

One of the chief problems in elucidating the mechanism of somatic hypermutation has been the absence of an in vitro system for analysis. Ongoing mutation of immunoglobulin V genes has been observed in B cell follicular lymphomas (110), although hypermutating cell lines derived from such lymphomas have not yet been described. Mutations have been detected during propagation of some mouse myelomas and pre–B cell lymphomas (111, 112), and it will be intriguing to ascertain whether the mutations detected in these tumors arose through a process akin to antibody somatic hypermutation. It may be that the recent improvements in the techniques of culturing and differentiating B lymphocytes in vitro (113, 114) will lead to effective systems for the study of hypermutation.

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ACCESSIBILITY CONTROL OF ANTIGEN-RECEPTOR VARIABLE-REGION GENE ASSEMBLY: Role of *cis*-Acting Elements

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ABSTRACT

Antigen receptor variable region genes are assembled from germline variable (V), diversity (D), and joining (J) gene segments. This process requires expression of V(D)J recombinase activity, and "accessibility" of variable gene segments to this recombinase. The exact mechanism by which variable gene segments become accessible during development is not known. However, several studies have shown that *cis*-acting elements that regulate transcription may also function to regulate accessibility. Here we review the evidence that transcriptional promoters, enhancers, and silencers are involved in regulation of accessibility. The manner in which these elements may combine to regulate accessibility is addressed. In addition, current and potential strategies for identifying and analyzing *cis*-acting elements that mediate locus accessibility are discussed.

1. INTRODUCTION

The genes that encode the variable region of antigen receptors are assembled during early lymphocyte development from germline variable (V), diversity (D), and joining (J) gene segments. Both immunoglobulin (Ig) and T cell receptor

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(TCR) variable region genes are assembled by an activity common to B and T cells referred to as V(D)J recombinase. The tissue-specific components of this recombinase are encoded by the recombinase activating genes (RAG) 1 and 2 (1). Assembly of Ig and TCR variable region gene segments is regulated in a tissue-, lineage-, and stage-specific fashion. Given that the V(D)J recombinase is common to B and T cells, direction of its activity during development must be regulated by the ability of given loci to serve as substrates for the recombinase, a concept referred to as "accessibility" (2, 3). The close correlation between transcriptional activation of germline variable region gene segments and recombination of these segments has led to the notion that *cis*-acting transcriptional regulatory elements may be involved, either directly or indirectly, in regulating accessibility. Antigen receptor gene assembly and accessibility control have been discussed in depth in recent reviews, and readers are referred to these for a more general discussion of particular aspects of these topics (2–4). In this review we focus on analyses of V(D)J recombination substrates in which putative *cis*-acting regulatory elements have been incorporated, and on gene targeted mutational analyses in which such elements are altered in the endogenous Ig or TCR loci. We also discuss the advantages and limitations of these approaches, and the need to identify additional types of elements involved in accessibility control.

2. V(D)J RECOMBINATION AND ACCESSIBILITY CONTROL

2.1 *Overview of the Mechanism of V(D)J Recombination*

Variable region gene segments are flanked by conserved recombination signal sequences (RSS), which consist of a heptamer, a spacer of 12 or 23 bp, and a characteristic AT-rich nonamer (5). In brief, the V(D)J recombination reaction involves recognition of the participating RSS, introduction of a double strand break (DSB) between the coding portion and RSS of two participating gene segments, and the subsequent precise joining of the RSS and the usually imprecise joining of the coding sequences (which may involve addition or deletion of nucleotides). The *cis*-acting RSS, which are conserved between Ig and TCR genes, are required and sufficient to target the common V(D)J recombinase to the adjacent coding segments (6, 7). While the RSS seem unlikely to be involved in mediating accessibility, they are clearly involved in directing the V(D)J recombination reaction. Variable gene segments capable of being joined efficiently must be flanked, respectively, by RSS with 12 and 23 base pair spacers (12/23 rule) (8). This joining specificity limits the types of accessible gene segments that may join with one another. Moreover, the nucleotide sequence

composition of the RSS or proximal coding sequences may affect the ability of individual variable region gene segments to recombine (9–11). Such sequence variation within a given accessible locus has been argued to be involved in determining the rearrangement frequency of certain variable region gene segments and the predilection for inversional vs deletional recombination of Ig heavy chain (HC) D segments (9, 11, and references within).

V(D)J recombination requires prelymphocyte-specific activities, which provide specificity in the initiation of the reaction by introduction of specific DSBs, and more generally expressed activities that appear to function in joining the broken ends. The products of RAG 1 and 2 appear to be the only required prelymphocyte-specific components of the V(D)J recombinase (1). Simultaneous expression of transfected RAG 1 and 2 genes is sufficient to generate V(D)J recombinase activity in all tested nonlymphoid mammalian cell lines. Furthermore, mice deficient in either gene product are blocked in early B and T cell development due to failure to initiate V(D)J recombination (12, 13). Recent in vitro studies suggest that RAG proteins are specific V(D)J recombinase components that function to recognize RSS and introduce DSBs (14). Therefore, accessibility control in vivo must direct the specific recognition and cutting activities of RAG 1 and 2 to desired sets of RSS. Potential mechanisms that may facilitate interaction of RAG proteins with particular RSS once a locus is accessible remain to be elucidated. In particular, it will be of interest to determine whether additional proteins interact with RAG proteins and *cis*-acting sequences, thereby targeting recombinase activity to appropriate loci.

The V(D)J recombination reaction also appears to employ generally expressed components. Analyses of severe combined immunodeficient (SCID) mice and of radiosensitive hamster cell lines have led to the identification of several ubiquitiously expressed proteins that may function to repair DSBs introduced during the V(D)J recombination reaction (15). One of these, the DNA-PK complex, is composed of three subunits including the DNA end-binding components, Ku70 and Ku80, and the catalytic domain DNA-PKcs. Cell lines deficient in Ku80 or DNA-PKcs exhibit defects in general DSB repair and are similarly defective in repairing the DSBs generated in V(D)J recombination. Additional components of the V(D)J recombinase remain to be identified but would likely include, among many others, ligases, polymerases, and perhaps factors involved in mediating initial accessibility. It is unlikely that the currently defined proteins involved in the V(D)J recombination reaction are directly involved in mediating locus accessibility. However, variations in their expression levels may influence this process, for example, by influencing the ability of cells to complete the reaction once initiated.

2.2 *Regulation of Antigen Receptor Gene Assembly During Lymphocyte Development*

Lymphocyte development is a complex, ordered process regulated by the progressive assembly and expression of antigen receptor genes in appropriate lymphoid cell types. It is believed that this ordered process evolved, at least in part, to facilitate regulation of variable region gene assembly and expression. Such regulation ensures that V(D)J recombinase–mediated rearrangements occur only in appropriate cell types and loci, and the regulation helps to ensure the specificity of the immune response.

Antigen receptor gene assembly occurs only in lymphoid cells. As the RAG 1 and 2 genes (and as a result V(D)J recombinase activity) are expressed almost exclusively in early B and T cells, their limited expression can be considered the major factor in this restriction. However, even if RAG 1 and 2 gene expression did occur in nonlymphoid cells, it is unlikely that there would be any significant level of endogenous antigen receptor gene segment rearrangement due to lack of accessibility. In this context, expression of transfected RAG 1 and 2 in nonlymphoid cells leads to rearrangement of artificially accessible recombination substrates but does not appear to lead to rearrangement of endogenous antigen receptor loci (1). Thus, antigen receptor genes appear to be entirely "closed" in nonlymphoid cells and are "opened" only in particular subsets of developing lymphocytes. Within the T and B cell lineages, complete assembly of TCR genes occurs only in T cells, and complete assembly of Ig genes occurs only in B cells. This restriction appears to occur primarily at the V-to-DJ rearrangement step as Ig HC D-to-J rearrangements can occur in T cells (16 and references within).

Within a given lymphoid lineage, the V(D)J recombination process is stage specific and can be allelically excluded. In B cells, Ig HC variable region genes are usually assembled prior to those of Ig light chain (LC) genes. Furthermore, assembly of Ig HC variable region genes occurs in an ordered sequence with D_H-to-J_H rearrangements preceding V_H-to-DJ_H rearrangements. Expression of an Ig Hμ chain gene from a productive V_HDJ_H rearrangement appears to generate a signal that halts V_H-to-DJ_H rearrangement of the other Ig HC allele, a process that is likely important in effecting HC allelic exclusion. This signal may also facilitate the onset of Ig LC V-to-J joining, although it is not yet clear if this is a direct effect or an indirect effect due to progression along the differentiation pathway (4). With respect to Ig LC genes, κ variable region genes are usually assembled prior to λ variable region genes, either through a specifically regulated mechanism, or due to stochastic competition mechanisms in which the κ locus is a better overall substrate for recombination than the λ locus (17–19). Once an Ig LC is paired with

an Ig HC to generate a complete Ig molecule, further assembly of LC variable region genes is halted, a process that may ensure LC allelic and isotype exclusion. The latter process may involve both a downregulation in V(D)J recombinase expression and a decreased accessibility of the LC variable region genes. TCR genes appear to follow an analogously ordered program of rearrangement and expression. For example, TCRβ variable region genes are assembled by sequential D_β-to-J_β and V_β-to-DJ_β rearrangements, and they are expressed prior to assembly of TCRα chain variable region genes from component V_α and J_α segments (20). Expression of a TCRβ chain results in signals that allellically exclude the other TCRβ allele by inhibiting the V_β-to-DJ_β-rearrangement step while leading to the rearrangement of TCRα loci (21, 22, 22a). As with lineage specificity, allelic exclusion of the TCRβ and Ig HC loci appears to occur at the V-to-DJ step of rearrangement. The nature of the sequences that regulate V-to-DJ rearrangement remains largely unexplored.

In summary, expression of a common V(D)J recombinase is activated early in lymphocyte differentiation and is then directed to rearrange certain gene loci or portions of these loci, to stop rearranging these loci, and then to begin rearranging additional loci. These precisely regulated steps must be due primarily to the differential accessibility of the particular loci to the common V(D)J recombinase. During prelymphocyte development, changes in accessibility appear to be regulated, at least in part, by signals generated as a result of the expression of individual chains of antigen receptors. None of these control processes appears absolute. Ig LC rearrangement has been observed in the absence of Ig HC gene expression, and TCRα rearrangement has been observed in the absence of TCRβ expression (3). Likewise, exceptions to allelically excluded rearrangement have been noted. Whether these represent background in the system, the existence of other developmental rearrangement programs, or both remains to be determined.

2.3 *Correlates of Accessibility in Endogenous Loci*

The mechanisms by which antigen receptor loci are made accessible to the V(D)J recombinase activity are not known. However, transcriptional activation, chromatin structure, and methylation may be involved in this process. Transcriptional activation is associated with changes in chromatin structure in endogenous loci (23). Similarly, changes in chromatin structure, as measured by DNase I hypersensitivity, have been associated with variable gene segments of endogenous loci and chromosomally integrated recombination substrates that are undergoing recombination (24, 25). However, it is unclear to what extent changes in chromatin are required for, or are a result of, recombinational accessibility.

Modification of genes by methylation of CpG islands affects transcriptional activity (26). The exact mechanism by which this occurs is not known but may involve inhibition of binding of *trans*-acting factors to methylated regions of DNA. Studies employing recombination substrates show a direct correlation between hypomethylation and accessibility to the V(D)J recombinase (27, 28). Although this correlation is intriguing, it is unclear whether hypomethylation is required for or is the result of making the locus accessible to the V(D)J recombinase. A single study indicates that recombination is blocked if substrates are methylated prior to introduction into a cell line that has V(D)J recombinase activity (29). However, the extrachromosomal recombination substrates employed in this study may be under different accessibility constraints than are integrated substrates or endogenous loci. Although it remains unclear whether hypomethylation is required for accessibility to the V(D)J recombinase, at least in some contexts hypomethylation per se is not sufficient for this process (30). In addition, methylation affects chromatin configuration; therefore, it is difficult to determine an effect of methylation independent of chromatin configuration (29, 31).

Active transcription of germline antigen receptor loci occurs at a time when these loci are undergoing recombination (3). Germline transcripts initiate upstream of the Ig HCμ constant (C) region gene and upstream of the J_H proximal DQ52 gene segment when the Ig HC locus is undergoing rearrangement (30, 32, 33). Transcription initiates from the J_κ-C_κ locus at the time of κ LC rearrangement (34). Likewise, germline transcripts have been observed from variable region gene segments coincident with their rearrangement (35–38). The best example of this is the TCRγ locus, where activation of V segment germline transcription is tightly correlated with the ordered rearrangement of these segments during development (35). Allelic exclusion of TCRβ rearrangement by an lck or TCRβ chain transgene is associated with a decrease in V_β germline transcription (39). Although the association between germline transcription and V(D)J recombination potential is quite compelling, the significance of these transcripts in this process is not currently known. Transcription could be required for, or could be a result of, an accessible locus. Germline transcripts also could be translated into proteins that regulate accessibility for subsequent steps of rearrangement (32, 40). Although possibly required, transcription initiated from V and D gene segments per se is not sufficient for rearrangement of these segments (41, 42).

The correlation between transcriptional activation and rearrangement of variable gene segments during development has led to the notion that elements regulating transcription may also regulate V(D)J recombination. At the simplest level eucaryotic gene transcription is regulated by *cis*-acting promoter

and enhancer elements (43). Transcriptional promoters are regions that lie immediately upstream of genes and serve as initiation sites for transcription by mediating attachment of the transcription complex. Promoter elements function in a distance- and orientation-dependent manner. Enhancer elements augment the level of transcription initiated from promoters. The exact mechanism by which they achieve this is unclear; however, they appear to be able to function in an orientation-independent manner over large distances. Transcriptional regulation of antigen receptor loci by promoters and enhancers exhibits tissue, lineage, and stage specificity, and these elements serve as likely candidates for the similar regulation of variable gene recombination (44–53). Although transcriptional activation may be an important mechanism by which these elements mediate accessibility, the inability of transcription per se to result in recombination implies that additional processes may be regulated by these elements. These processes may include changes in methylation status or chromatin structure independent of transcriptional regulation (54).

3. EXPERIMENTAL SYSTEMS FOR STUDYING ACCESSIBILITY

3.1 *Overview of Approaches*

Recombination of germline antigen receptor variable gene segments requires expression of the common V(D)J recombinase and accessibility of the target RSS to the recombinase. Maintenance of accessibility of loci undergoing rearrangement, and inaccessibility of loci not undergoing rearrangement, is important in the developmental regulation of variable gene assembly. Experiments designed to evaluate *cis*-acting elements that regulate accessibility have focused primarily on transcriptional enhancers, with some analyses of the role of promoters and silencers. Recombination substrates with putative regulatory elements have been analyzed either as transgenes or in cell lines that express V(D)J recombinase activity. In addition, endogenous loci have been analyzed in which *cis*-acting elements have been deleted by gene targeting.

3.2 *V(D)J Recombination Substrates*

Both extrachromosomal and chromosomally integrated recombination substrates have been assayed for V(D)J recombination potential. However, chromosomally introduced substrates are more likely to have accessibility constraints similar to those of endogenous genes; accordingly, most accessibility analyses have used integrated substrates. A V(D)J recombination substrate requires a minimum of two RSS with 12 and 23 bp spacers required for recognition by the V(D)J recombinase. However, many stably introduced substrates have

been large in size and have included one or more V, D, and J gene segment(s) and a C region gene (55–61). Putative regulatory elements have been manipulated in such substrates, and the effects on V(D)J recombination determined. Although analysis of V(D)J recombination by this approach has provided much useful information, results of these studies can be confounded by a variety of potential nonphysiologic regulatory effects due to integration site, copy number, and proximity of plasmid DNA (62). Such effects may be limited in future studies by employing strategies that ensure single copy integration, deletion of plasmid DNA, limitation of local DNA effects by the use of insulators, or by further increasing the size of substrates (63).

3.3 *Cell Transfection Studies*

Cell culture analyses of V(D)J recombination have employed cell lines with constitutive V(D)J recombinase activity (25, 55, 64, 65). These systems have been used for assays of transient recombination substrates; such studies indicated that accessibility of small, extrachromosomal substrates correlated with methylation status but not transcriptional activity (29, 66). Additional analyses of stably integrated substrates in Abelson murine leukemia virus (A-MuLV)-transformed pre-B cell lines transformants suggested a role for enhancer elements in mediating accessibility (55). However, analyses of stably integrated substrates in constitutively expressing lines can be complicated by a number of potentially nonphysiologic factors. For example, cell lines with constitutive V(D)J recombinase activity may exhibit clonal variation in expression of RAGs and, potentially, other factors that influence V(D)J recombination (67). In addition, in such cell lines, it is often difficult to determine whether recombination of substrates occurs prior to or after integration into the genome. This is an important point as accessibility of integrated vs extrachromosomal substrates likely displays different regulatory constraints. To circumvent some of these problems, RAG 1 and 2 genes under the control of the heat shock promoter have been stably integrated into a B cell lymphoma line (65). Heat shock of these cells, termed DR37 cells, resulted in a transient increase in the levels of RAG 1 and 2 expression and the coincident induction of V(D)J recombinase activity. Thus, unrearranged substrates can be integrated into the DR37 line prior to V(D)J recombinase induction and their rearrangement potential assayed subsequent to induction (65).

Recombination substrates introduced into the DR37 cell line have exhibited a clear dependence on enhancer elements for V(D)J recombinational accessibility (65). In particular, an enhancerless TCRβ recombination substrate stably transfected into this cell line failed to undergo V(D)J recombination subsequent to induction (65). However, insertion of the Ig HC intronic enhancer, Ig LC κ intronic enhancer, and the simian virus 40 enhancer into the same

substrate promoted its rearrangement in this system, indicating that even non-lymphoid enhancer elements can promote V(D)J recombinational accessibility (65). However, only a small percentage of the integrated substrates underwent rearrangement in these analyses; assuming uniform induction of V(D)J recombinase activity in all cells of the population, this finding indicates that the recombination substrates tested either lack important *cis*-acting elements or that the DR37 cell line lacks *trans*-acting factors necessary for optimal substrate accessibility. Investigation into these two possibilities may lead to additional insights into the regulation of accessibility.

V(D)J recombinase inducible cell lines that represent stages of early B and T cell development will be needed to expand the utility of the cell transfection approach for studying stage-specific factors that regulate accessibility. In this regard, novel temperature sensitive A-MuLV-transformed pre-B cell lines in which V(D)J recombinase is upregulated at the nonpermissive temperature have been described (68). At the permissive temperature, the virus inhibits RAG expression and Ig LC κ rearrangement. At the nonpermissive temperature RAG levels are upregulated and κ LC rearrangement is observed. Accessibility of endogenous loci and introduced recombination substrates could be analyzed in such lines.

3.4 *Analysis of Transgenic Recombination Substrates*

Constructs stably introduced into cell lines have exhibited different requirements for *cis*-acting transcriptional regulators when compared to analysis of these same constructs as transgenes (69–71). Among other things, these differing requirements may be due to modifications such as gene methylation occurring during development (72). Unlike substrates in cell lines, transgenic recombination substrates should be subject to developmental modifications and to the effects of developmentally regulated *trans*-acting factors. In particular, transgenic substrates are present in the precise cell type of interest and therefore should be exposed to normal levels of V(D)J recombination activity. However, interpretation of these studies still can be complicated by the nonphysiologic effects of integration site and copy number.

Several transgenic substrates have exhibited a clear dependence on enhancer elements for accessibility to the V(D)J recombinase. The Ig HC intronic enhancer, Ig LC κ intronic enhancer, and the TCRα and β enhancers are capable of mediating accessibility of a TCRβ transgenic recombination substrate (41, 56, 73). The TCRδ and α enhancers are capable of mediating accessibility of a human TCRδ transgenic recombination substrate (60, 74). The chicken Ig LC λ enhancer mediates accessibility of a chicken Ig LC λ transgene (58), and the rabbit Ig LC κ intronic enhancer mediates accessibility of a rabbit Ig LC κ transgene (57). The inability of most of these transgenes to undergo

efficient recombination in the absence of enhancer elements underscores the importance of enhancers in mediating accessibility (56–58). An interesting exception to this enhancer dependency occurs in a human TCRδ transgene (60). The first rearrangement step (V-to-D) of this transgene occurs in the absence of the defined enhancer element. This may represent an exception to the general requirement for enhancer elements. Alternatively, another enhancer element may be present in this substrate that mediates V-to-D rearrangement without allowing for subsequent VD-to-J rearrangement. The possible role of multiple enhancer elements in mediating locus accessibility is discussed below.

Preliminary evidence from transgene studies has suggested that enhancer elements may be responsible for dictating the stage specificity of V(D)J recombination. Recombination of the endogenous TCRα locus occurs at a later developmental stage than does recombination of the TCRβ, γ, and δ loci (20). TCRβ or TCRδ transgenes with a TCRα enhancer also rearrange later in development than do the same transgenes with the TCRβ or TCRδ enhancer, respectively (73, 74). Additional work, including targeted replacement of enhancer elements in endogenous loci, will be required to clearly establish the role of enhancer elements in mediating stage-specific control of recombination and to identify other elements that may modify their action.

Some transgenic recombination substrates also exhibit lineage-specific control, and they therefore are likely to contain *cis*-acting elements responsible for this control (27, 41, 56, 60, 73, 75). Among these, the lineage-specific steps are best characterized for the TCRβ and TCRδ transgenes (41, 56, 60, 73). In the TCRβ locus, D-to-J rearrangement occurs prior to V-to-DJ rearrangement. Conversely, in the TCRδ locus V-to-D rearrangement occurs prior to VD-to-J rearrangement. In both cases lineage-specific regulation of recombination appears to be exerted at the final recombination step. The TCRβ transgene undergoes D-to-J rearrangement in B and T cells and V-to-DJ rearrangement only in T cells; the TCRδ transgene undergoes V-to-D rearrangement in B and T cells, and VD-to-J rearrangement only in T cells (41, 56, 60, 73). The *cis*-acting elements capable of imparting lineage specificity have not yet been identified. However, the TCRβ transgenic recombination substrate undergoes complete V(D)J rearrangement exclusively in T cells when the Ig HC intronic enhancer, TCRβ enhancer, or TCRα enhancer are incorporated into the substrate, suggesting that these enhancer elements cannot independently impart lineage specificity (41, 56, 73). TCRβ transgenic recombination substrates that appear to undergo allelic exclusion in the presence of a rearranged TCRβ transgene have been described (59, 61). In the future, these substrates should serve as useful reagents to identify *cis*-acting elements involved in allelic exclusion.

Analysis of transgenic recombination substrates has suggested that transcriptional promoters and silencers may also be involved in regulating accessibility of these substrates. In promoter elements, mutation of the octamer region abolishes the ability of these elements to initiate transcription (45). Recombination of a chicken Ig LC λ transgene depends on the integrity of the V gene segment promoter (58). Compared to mice with the wild-type transgenes, V-to-J rearrangement was markedly diminished in the majority of mice containing a transgene with a mutated octamer site in the V segment promoter. The Ig LC λ transgene was not transcribed in either bone marrow or spleen cells of these mice. Transcriptional silencer elements are *cis*-acting elements that can inhibit transcription in a distance- and orientation-independent manner. Transcriptional silencer elements have been identified in the TCRα locus (76), the mouse Ig LC κ locus (77), and the chicken Ig LC λ locus (58). Deletion of the silencer in the chicken Ig LC λ transgene results in a fivefold increase in the level of rearrangement as compared to the wild-type transgene (58). Transcription of this recombination substrate is also increased. Although these studies imply that promoter and silencer elements may regulate accessibility, further analysis will be required to clearly establish the role of these elements in this process.

3.5 *Deletion of Elements in Endogenous Loci*

Recombination substrate findings cannot unequivocally establish a role for enhancer elements in mediating accessibility of endogenous antigen receptor loci. Recently, direct evidence for such a role has come from application of gene targeting to the study of *cis*-acting elements (18, 22, 30, 78). Gene targeting allows elements to be replaced, deleted, exchanged, or subtly mutated in the endogenous loci. Many of the initial gene targeting studies of *cis*-acting elements, while providing intriguing results, were hampered by possible nonphysiologic effects caused by the introduction of a constitutively expressed drug resistance gene. In these experiments, the role of the targeted element was often difficult to determine, because the introduced drug resistance gene could itself affect transcription and rearrangement of the targeted locus. Contrasting results obtained in the presence and absence of drug resistance genes have lent insight into the possible presence of additional elements (see below). However, these contrasting results also mandate deletion of inserted drug resistance genes before conclusions can be drawn about the function of deleted elements. For this reason, alternate strategies have been used to allow initial replacement of an element by a drug resistance gene, followed by the subsequent deletion of the drug resistance gene. One strategy, called hit-and-run targeting, involved design of a complicated targeting construct that would result in a clean deletion of the element of interest (78). A more widely used approach utilizes the site specific cre recombinase and its *loxP* target sites, both originally isolated from

phage P1 (79, 80). Initially the *cis*-acting element is replaced by a drug resistance gene flanked by *loxP* sites. Subsequently, cre-mediated deletion of the drug resistance gene leaves a single *loxP* site in place of the targeted element. Replacement of enhancers with a neo^r gene and/or with a *loxP* site has provided useful information about the role of enhancers in mediating accessibility of endogenous antigen receptor loci.

The Ig HC intronic enhancer (E_μ) lies between the J_H gene segments and the C_μ gene (46, 47) and is closely associated with two matrix attachment regions (MARs) (81). In the following discussion, we do not distinguish the core E_μ element from the core element plus the adjacent MAR sequences as both were mutated. Targeted mutation studies have definitively shown that E_μ plays a role in regulating rearrangement of the HC locus. In one study, hit-and-run replacement of E_μ with a short oligonucleotide resulted in slightly diminished D_H-to-J_H rearrangement (70% of normal) but more substantially inhibited V_H-to-DJ_H rearrangement of the targeted allele (78). In a second study, replacement of E_μ with a neomycin resistance (neo^r) gene resulted in a dramatic decrease in the ability of the J_H locus to undergo recombination (30). Notably, insertion of the neo^r gene upstream of E_μ, without deletion of endogenous sequences, resulted in a similar *cis*-acting inhibition of J_H rearrangement (30). Thus, the neo^r resistance gene may be competing for the activity of additional elements responsible for regulating accessibility of the D_H and J_H gene segments. Similar findings have been made with respect to enhancer elements within the Ig LC κ locus (see below), and the locus control region (LCR) in the β-globin locus. Insertion of a neo^r gene in the LCR blocks β-globin gene expression, possibly by outcompeting the endogenous globin promoters for the enhancer activity of the LCR (82).

Three additional enhancers have been identified within the Ig HC locus (53, 83–85). A fragment encompassing the J_H proximal DQ52 segment and the region immediately 5′ to DQ52 has promoter and enhancer activities in early B cells (83). Given the location and activity of this element, it has been suggested that, prior to DJ_H rearrangement, the DQ52 promoter/enhancer may compete more efficiently than upstream elements for interaction with E_μ, conferring accessibility to the J_H region. Any DJ_H rearrangement not involving DQ52 itself would delete this element, thereby freeing E_μ to interact with upstream regulatory elements that initiate V_H-to-DJ_H rearrangement. This proposed interaction between the DQ52 promoter/enhancer and E_μ could be responsible for the ordered rearrangement observed in the Ig HC locus (D_H-to-J_H followed by V_H-to-DJ_H) (83). Two additional enhancer sequences have been shown to lie at the 3′ end of the Ig HC locus. One of these is a weak enhancer that lies immediately downstream of Cα (84). A

much stronger enhancer ($3'E_H$) lies approximately 16 kb downstream of the Cα gene (53). B cells in which a 5-kb region spanning the $3'E_H$ was replaced with a neo^r gene have defects in germline Ig HC constant region gene transcription and class switch recombination to certain Ig HC constant region genes that lie as much as 150 kb upstream of this region (86). However, such mice show no major impairment in V(D)J recombination. These data imply that the $3'E_H$ is not required for variable region gene assembly; however, in this analysis a decrease in efficiency of recombination would not be detected. Of note, it was speculated that the effects of the $3'E_H$ replacement may have resulted from competition of the inserted neo^r gene for additional enhancer elements of an LCR. In this context, a region encompassing as much as 20 kb downstream of the $3'E_H$ contains additional enhancer elements and has been implicated as having LCR-like activities (85). Such a region could be involved in long-range control of the accessibility of the Ig HC locus.

The IgL κ locus contains two defined enhancer elements. The intronic enhancer (iE$_\kappa$) lies between the J$_\kappa$ region and the C$_\kappa$ gene (87–89). The iE$_\kappa$ element becomes transcriptionally active during the preB-to–B cell transition (50, 52, 90). This stage specificity has been attributed to the presence of a 10-bp motif termed κB, that binds the NF-κB protein, itself constitutively active in mature B cells and plasma cells. The observation of high-level κ LC production in the S107 plasmacytoma line (52), which does not express NF-κB, led to the identification of a second enhancer element, the $3'$ κ LC enhancer ($3'E_\kappa$), which lies approximately 9-kb $3'$ of the C$_\kappa$ gene (91). Various studies have suggested that $3'E_\kappa$, either alone or in synergy with the iE$_\kappa$ element, is important for high level expression of a rearranged kappa locus (91–97, and references within). While these studies left unclear the role of $3'E_\kappa$ in early B cells, they suggested that $3'E_\kappa$, either alone or in combination with iE$_\kappa$, is important for high level expression of a rearranged kappa locus.

Gene-targeted replacement of the iE$_\kappa$ (18) or iE$_\kappa$ and the $5'$ MAR (Y Xu, F Alt, D Baltimore, unpublished) with a neo^r gene appears to block rearrangement of the J$_\kappa$ locus completely, whereas insertion of a neo^r gene just downstream of iE$_\kappa$ reduces but does not completely inhibit rearrangement (18). However, replacement of the iE$_\kappa$ and $5'$ MAR with a single *loxP* site diminishes but does not completely inhibit J$_\kappa$ rearrangements (Y Xu, F Alt, D Baltimore, unpublished). These data imply that the iE$_\kappa$ promotes J$_\kappa$ rearrangement but that additional elements, whose function is blocked by insertion of the neo^r gene, also promote rearrangement in the absence of iE$_\kappa$. Replacement of the $3'E_\kappa$ with a neo^r gene also results in a substantial inhibition of J$_\kappa$

rearrangement (J Gorman, F Alt, unpublished). It is not yet clear whether this effect is due to deletion of the $3'E_\kappa$ element, competition for the activity of some other element required for J_κ rearrangement, or some combination of these.

The TCRβ locus contains a single defined enhancer element (E_β), which is in the 3′ region of the locus immediately upstream of $V_\beta 14$ gene segment (98). Deletion of E_β leads to a marked decrease in rearrangement, indicating that this element has a major role in promoting accessibility of the TCRβ locus (J-C Bories, F Alt, unpublished; P Ferrier, personal communication). Furthermore, replacement of E_β with E_μ restores rearrangement (perhaps at reduced levels) in T cells but does not lead to significant rearrangement of the locus in B cells, implying that other elements in the TCRβ locus may be involved in determining the lineage specificity of its rearrangement (J-C Bories, F Alt, unpublished). In another study, a large deletion/neo^r gene replacement of the TCRβ region that removed the constant regions but retained E_β also blocked V(D)J recombination in the locus. In this case, the inhibition could have resulted from effects of the inserted neo^r gene on E_β, deletion (or inhibition) of some unknown required element, or both (22).

Future use of targeting strategies should be initially aimed at defining all of the elements involved in mediating locus accessibility. These will likely include combinations of transcriptional promoters, enhancers, and silencers in addition to novel elements that may or may not have a role in transcriptional regulation. Partial effects seen upon deletion of individual elements suggest that accessibility of antigen receptor gene loci is regulated by multiple elements. Therefore, understanding the regulation of accessibility of these loci will likely require the simultaneous deletion of multiple elements within a single locus. Experiments involving replacement of elements in one locus with similar elements of another locus may lend insight into stage and lineage-specific regulation of V(D)J recombination.

3.6 *Studies of Trans-Acting Factors*

Regulation of transcription is mediated in part by the interaction of *trans*-acting DNA-binding proteins with specific motifs in promoter and enhancer elements. Regulation of accessibility by enhancer elements likely involves similar interactions. Although enhancers are defined by their ability to augment the level of transcription initiated from promoter elements, dissection of motifs within enhancers has revealed a more complex regulation including both positive and negative components (99, 100). Many of these motifs are present in the regulatory elements of disparate genes and bind to ubiquitous *trans*-acting DNA-binding proteins; yet, transcriptional regulation can be restricted in a tissue-, lineage-, and stage-specific manner. This has led to the notion of "combinatorial

regulation" of transcription in which the net regulatory effect is due to a unique combination of relatively ubiquitous *trans*-acting DNA-binding proteins that interact with the regulatory elements of a specific gene (99, 100).

Motifs within E_μ that potentially bind to *trans*-acting DNA binding proteins have been mapped in detail (45, 100). A 220-bp region of E_μ that defines the minimal region required for transcriptional activity was sufficient to mediate accessibility of a TCRβ recombination substrate in a V(D)J recombinase inducible B cell line (65). In an attempt to address the role of individual binding motifs, several mutated forms of E_μ have been analyzed in the transgenic TCRβ recombination substrate (42). The E-box motif, μE3, in conjunction with motifs upstream of μE3, appears to be essential for E_μ-mediated accessibility to the V(D)J recombinase. In contrast to the role for the octamer site in promoter elements (58), the octamer site in E_μ does not appear to be required for this enhancer to mediate accessibility (42). The κB motif of iE_κ appears to be essential for the transcriptional activity of this enhancer (45). The κB motif is also necessary for optimal function of the iE_κ in mediating accessibility of a TCRβ recombination substrate in a recombinase inducible cell line (100a). However, the κB motif is not sufficient to mediate accessibility of this substrate. Mutation of the c-Myb binding site or the core binding factor (CBF) site within the TCRδ enhancer also results in decreased recombination of a human TCRδ transgene (C Hernandez-Munain, P Lauzurica, M Krangel, personal communication) (101, 101a). Additional work will be required in this area to define the roles of binding motifs in mediating accessibility.

Gene-targeted deletions of *trans*-acting factors that bind to specific motifs within enhancer and promoter elements have yielded much information about the roles of these proteins in lymphoid development. However, it has been difficult to use these studies to assign specific roles for these proteins, and their respective binding motifs, in regulating recombination. A detailed description of all of these studies is beyond the scope of this review. Therefore, we choose representative examples to illustrate the issues encountered with this approach. Disruption of the genes encoding the E2A (102, 103) or EBF (104) transcription factors results in a block in B cell development at a stage prior to Ig HC rearrangement. In each of these studies it is unclear if the failure to rearrange Ig HC genes is the cause of, or the result of, the developmental block. Mice deficient for Oct-2 (105) or NF-κB (106, 107) expression appear to display normal B cell development, including rearrangement of Ig HC and Ig LC genes. It is clear from these studies that Oct-2 and NF-κB are not absolutely required for Ig gene rearrangement. However, it remains possible that Oct-2 and NF-κB normally function to mediate accessibility and that in their absence redundant family members function in this capacity.

In conclusion, the functional promiscuity and redundancy exhibited by many families of DNA-binding proteins will make it difficult to apply targeting strategies to determine if these proteins are directly involved in mediating antigen receptor accessibility. An approach of directly analyzing motifs in elements in the endogenous locus could be problematic as deletions of entire elements, such as E_μ or iE_κ, reduce but do not ablate rearrangement of their associated loci. Once all of the essential elements required for accessibility of an endogenous loci have been identified, it may be possible to conceive of strategies to assay for the function of particular motifs within a given element. At present, analysis of recombination substrates containing enhancers with mutated motifs, while not clearly physiologically relevant, can provide initial information about how particular motifs may function in mediating V(D)J recombinational accessibility.

3.7 *In Vitro Analysis of Accessibility*

The initial steps of recombination can be carried out in vitro (14); further work may yield an in vitro system in which the entire reaction can be carried out. Of relevance, in vitro analyses have been used to study the regulation of initiation of transcription (108). Such systems have been used to investigate the role of chromatin configuration in regulating accessibility to transcriptional machinery. As the initial steps of V(D)J recombination can now be carried out in vitro, it may be possible to extend this approach to analyses of the determinants of accessibility.

4. CONCLUSIONS AND FUTURE DIRECTIONS

Antigen receptor variable region gene assembly is a complex, developmentally regulated process that is under tissue-, lineage-, and stage-specific control, and it thus has many parallels with transcriptional control (44–53). Recombinational control is mediated by differential accessibility of antigen receptor loci to the V(D)J recombinase as dictated at least in part by *cis*-acting elements that function in transcriptional regulation of antigen receptor genes. Experiments employing recombination substrates and deletion of elements in endogenous loci have implicated transcriptional enhancers, promoters and silencers in the control of variable gene accessibility during development. The exact mechanism by which these elements mediate accessibility has not been determined. They may function to control accessibility by regulating transcription, altering chromatin structure, affecting gene methylation, and/or by recruiting components of the V(D)J recombinase.

The role of transcriptional enhancers in the regulation of V(D)J recombination has been analyzed in most detail. These analyses suggest that accessibility

of antigen receptor loci may rely on the activity of multiple elements within a given locus. Multiple enhancer elements may functionally or physically interact to allow full accessibility of an entire locus or regions of a locus. Such a possibility is indicated by the finding that deletion of the E_μ or iE_κ diminishes but does not abolish rearrangement of the linked J loci (18, 30, 78; Y Xu, FW Alt, D Baltimore, unpublished). Particular enhancer elements also may function to allow for accessibility of different regions of given antigen receptor loci (e.g. V vs J regions), for example, allowing for regulation of V-to-DJ vs D-to-J rearrangement steps. Such regional control could be due to the division of antigen receptor loci into distinct regions of chromatin (a proposed function of MARs), and the presence of elements that regulate accessibility within each region. Alternatively, elements need not be physically contained within the regions they regulate, but rather they may regulate these regions from a distance. This may occur, for example, due to a requirement for a coupled interaction between a promoter and a distant enhancer via a *trans*-acting factor. Such interactions could be further regulated via competition mechanisms in which deletion of a particular cooperating element via a rearrangement event may allow a retained element to activate a more distal element in a different region. Such a general model has been proposed to explain the regulation of D-to-J_H vs V_H-to-DJ_H rearrangement of the Ig HC locus (83). The notion that *cis*-acting elements may interact to regulate accessibility is consistent with the manner in which these elements have been proposed to function in the regulation of transcription (43).

Although transcriptional enhancer elements appear to play an important role in mediating locus accessibility, other transcriptionally active elements, such as promoters and silencers, also appear to function in this regard. The mechanism by which transcriptional promoters and silencers regulate accessibility, either independently or in combination with enhancers, is not currently known. Several antigen receptor loci contain MARs and LCRs (81, 85, 109–112). MARs are AT rich regions that are defined by their ability to bind the nuclear matrix. Although the exact function of MARs is unclear, they have been hypothesized to demarcate regions of chromatin, and they can contain regions that undergo base unpairing, and regions that mediate binding of topoisomerase II (109, 113). LCRs are defined by their ability to impart position independence and copy number dependence on expression of transgenes, and LCRs are thought to function by affecting chromatin structure over long distances (43). They may contain functionally distinct elements such as enhancers within their boundaries. LCRs are involved in the regulation of multigenic loci such as the β-globin locus (43). Regulatory elements within the locus may compete for interaction with enhancer components of the LCR (114). Furthermore, the nature of this competition may change during development, resulting in

altered expression of genes within the locus. In this context, insertion of a drug resistance gene into the LCR of the β-globin locus results in transcriptional inactivation of β-globin genes (82).

Understanding the mechanism by which *cis*-acting elements regulate accessibility will require identification of all such elements within an antigen receptor locus and their systematic mutation alone and in various combinations. Such elements may include those that function in transcriptional regulation and/or mediate accessibility independent of transcriptional regulation. A potential example of the latter is an element in the Ig LC κ locus that has as yet no defined transcriptional activity but appears to be involved in regulating accessibility of the locus (115, 116). Other approaches for identification of additional elements may include, for example, isolation of V(D)J recombination substrate regions that determine substrate accessibility. Elements can be identified by looking for distinct DNase 1 hypersensitivity sites that appear as regions of the loci become accessible to the V(D)J recombinase. In addition, potential novel elements can be identified by comparing antigen receptor gene sequences across species looking for noncoding regions that are highly conserved. Any strategy aimed at detection of novel elements involved in regulating accessibility will have to take into account the possible long-range effect of these elements. In this regard it has been demonstrated that replacement of the $3'E_H$ with a neo^r gene can interfere with accessibility to the switch recombinase of constant region genes at a distance of up to 150 kb (86). This observation mandates that investigations aimed at identifying novel elements consider regions at great distances from the region being controlled.

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IMMUNOPHARMACOLOGY OF RAPAMYCIN[1]

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ABSTRACT

The potent immunosuppressive drugs FK506 and rapamycin interfere with signal transduction pathways required for T cell activation and growth. The distinct inhibitory effects of these drugs on the T cell activation program are mediated through the formation of pharmacologically active complexes with members of a family of intracellular receptors termed the FK506 binding proteins (FKBPs). The FKBP12 · FK506 complex specifically binds to and inhibits calcineurin, a signaling protein required for transcriptional activation of the interleukin (IL)-2 gene in response to T cell antigen receptor engagement. The FKBP12 · rapamycin complex interacts with a recently defined target protein termed the mammalian target of rapamycin (mTOR). Accumulating data suggest that mTOR functions in a previously unrecognized signal transduction pathway required for the progression of IL-2-stimulated T cells from G_1 into the S phase of the cell cycle. Here

[1]Abbreviations: AT, ataxia telangiectasia; CaN, calcineurin; cdk, cyclin dependent kinase; CLN, cyclin; CRC, calcium release channel; CsA, cyclosporin A; eIF, eukaryotic initiation factor; FKBP, FK506 binding protein; IFN, interferon; IL, interleukin; IP3R, inositol trisphosphate receptor; Kip, cyclin-dependent kinase inhibitor protein; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NFAT, nuclear factor of activated T cells; ORF, open reading frame; PHAS, phosphorylated heat-and-acid stable; PI 3-kinase, phosphatidylinositol 3-kinase; PI 4-kinase, phosphatidylinositol 4-kinase; PPIase, peptidyl-prolyl isomerase; RAP, rapamycin; RyR, ryanodine receptor; TcR, T cell receptor; TOR, target of rapamycin

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we review the immunopharmacology of rapamycin, with particular emphasis on the characterization of mTOR.

INTRODUCTION

The generation of immune responses to most antigenic stimuli is crucially dependent on an initial phase of T cell activation and proliferation. Activation of T cells is conventionally divided into two sequential stages. Prior to recognition of its cognate antigen, the mature T cell resides in the G_0 phase of the cell cycle. During the first stage of activation, contact with an antigen-presenting cell delivers the signals required for cell-cycle entry (G_0- to G_1-phase transition) and the expression of high-affinity receptors for T cell growth factors, including interleukin (IL)-2 and IL-4. The initial activation step also elicits the production of IL-2 and other growth-promoting cytokines by a subset of T cells within the activated population. The subsequent binding of T cell growth factors to their high-affinity receptors initiates the signaling events required for the progression of the G_1-phase T cell into S phase and, ultimately, into mitosis.

The bacterially derived immunosuppressants FK506 (tacrolimus, Prograf) and rapamycin (sirolimus, Rapamune) have proven to be powerful pharmacologic probes for the dissection of signal transduction events related to T cell activation and growth. Although both drugs bind to the same immunophilin receptor, the resulting immunophilin · drug complexes interfere with distinct intracellular signaling pathways in T and other types of cells. Treatment of T cells with FK506 specifically inhibits the activity of the Ca^{2+}-regulated serine-threonine phosphatase, calcineurin, an enzyme required for the transmission of T cell activating signals from the T cell antigen receptor (TcR). In contrast, the formation of immunophilin · RAP complexes in T cells interferes with the abilities of T cell growth factors to drive the progression of these cells from G_1 to S phase of the cell cycle. Recent studies have identified homologous target proteins for the immunophilin · RAP complex in yeast and mammalian cells. Ongoing studies with RAP as a probe are beginning to uncover a novel signal transduction cascade that may play a general role in the regulation of cell cycle progression in lymphoid and other eukaryotic cells.

DISCOVERY AND IN VIVO IMMUNOSUPPRESSION

Rapamycin (RAP), a lipophilic macrolide, was identified more than twenty years ago during antibiotic screening at Ayerst Research Laboratories. Produced by a strain of *Streptomyces hygroscopicus* isolated from a soil sample

obtained from the Vai Atore region of Easter Island (Rapa Nui) (1), RAP is a white crystalline solid (m.p. 183–185°C), virtually insoluble in water but readily soluble in ethanol, methanol, dimethylsulfoxide, and other organic solvents (2). Although lacking antibacterial activity, RAP is a potent inhibitor of yeast growth and a moderate growth inhibitor of filamentous fungi (2). It is most active against species of *Candida*, particularly *C. albicans*, and protects against systemic and vaginal candidosis in mice, without acute toxicity (LD_{50}: 597 mg/kg intraperitoneally) (3). Early mechanistic studies showed that concentrations of RAP as high as 1.0 μg/ml do not inhibit *C. albicans* growth during the first hour after addition to the growth media, but that concentrations as low as 5 ng/ml are growth-inhibiting after 90 min (4). In addition to fungicidal activity, RAP exerts tumoricidal activity (5), establishing that the antiproliferative effects observed in yeast can be extended to mammals. Labeling studies in *C. albicans* demonstrated that RAP strongly inhibits the incorporation of [^{32}P] phosphate into DNA and RNA (4), an indication that the drug exerts an effect upon the cell cycle. The structural characterization of RAP ($C_{51}H_{79}NO_{13}$; Figure 1) showed the molecule to be a mixture of two conformational isomers due to *cis-trans* rotation about an amidic bond in the 31-membered macrolide ring (6). The proposed chemical structure (6) was confirmed by the total organic synthesis of RAP (7–10).

The first demonstration of RAP's immunosuppressive activity was obtained from studies showing its inhibitory effects upon production of humoral IgE as well as its preventative effects in two animal models of human autoimmune disease, experimental autoimmune encephalitis and adjuvant arthritis

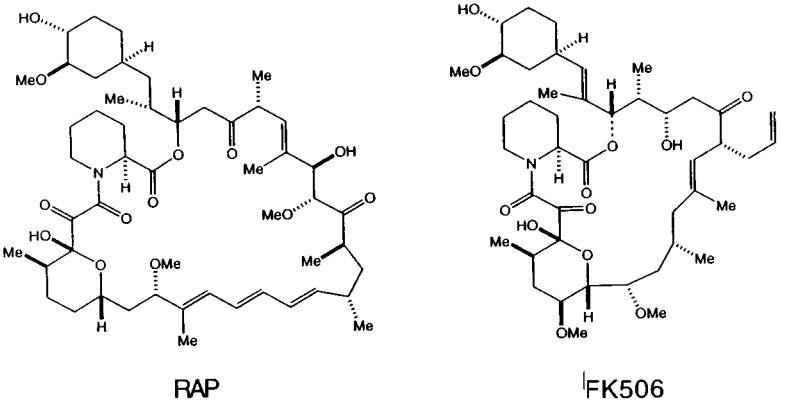

Figure 1 The chemical structures of rapamycin (*left*) and FK506 (*right*).

(11). However, the drug received more serious consideration as an immunosuppressant nearly a decade later, coincident with the discovery of FK506 at the Fujisawa Pharmaceutical Laboratories. FK506 ($C_{44}H_{69}NO_{12}$, Figure 1) was identified in a strain of *Streptomyces tsukabaensis* isolated from a soil sample taken from the Tsukuba region of Northern Japan during a screen for natural products that inhibit IL-2 production (12). FK506 was shown to be a powerful immunosuppressive agent as measured by its inhibitory effects in several immune function assays, including alloantigen-induced proliferation of lymphocytes, generation of cytotoxic T lymphocytes, expression of IL-2 receptors, and the production of T lymphocyte–derived lymphokines such as IL-2, IL-3, and IFN-γ (13). Interest in the therapeutic potential of FK506 mounted when it was discovered to be 100-fold more potent than cyclosporin A (CsA), the mainstay of transplant rejection drugs (13). The observations that the macrolactam rings of FK506 and RAP both contain the distinctive hemiketal-masked α, β-diketopipecolic acid amidic component (Figure 1) (14) and that FK506 is a more potent immunosuppressant than CsA provoked a resurgence of interest in RAP as a candidate immunosuppressive drug (15).

Although RAP and FK506 bind to the same family of intracellular receptors (see below), studies of the immunosuppressive activities of both agents in vitro (16, 17) quickly proved that the mechanism of RAP action is distinct from that of FK506. While the development of RAP as a therapeutic agent has lagged behind that of FK506, studies in both rodent and larger animal models have confirmed that RAP is a potent immunosuppressive compound. The therapeutic indices of RAP in various species, arranged in the order of greatest to least, are: mouse and rat, pig, monkey, and dog (18; reviewed in 19). In rodents, RAP is a potent inhibitor of the rejection of both xeno- (20) and allogeneic (21) skin grafts and, in highly histoincompatible heart grafts (18, 22), it significantly outperforms FK506 in terms of both potency and graft survival time (22). In histoincompatible renal transplants in pigs (18, 23, 24), rats (25), and primates (26), RAP has also demonstrated its effectiveness in preventing rejection. In mice, it is a more potent inhibitor of the graft-versus-host reaction than either FK506 or CsA. In the same species, RAP prevents host-versus-graft disease with higher potency than CsA, but not FK506 (27). Further studies have confirmed the initial finding (11) that RAP protects against autoimmune disease in rodent models. In the MLR/lpr mouse model of human systemic lupus erythematosus, RAP prolongs survival and prevents progression of the glomerulonephritis associated with the disease. In the nonobese diabetic mouse model of human autoimmune insulin-dependent diabetes mellitus, RAP prevents the onset of the disease whereas CsA is without effect (28). RAP also inhibits developing and established adjuvant-induced arthritis in the rat and has

significant inhibitory effects on the incidence and severity of collagen-induced arthritis in mice (28).

Relative to the severe renal side effects observed during therapy with CsA and FK506, the nephrotoxicity caused by RAP treatment is negligible (18, 28–30). However, it is not without serious side effects, particularly in larger animals. As observed with FK506 (31), RAP is extraordinarily toxic when administered to dogs, and even short, nonimmunosuppressive dosing regimens severely disturb gastrointestinal functions, producing diarrhea, vomiting, severe ulceration, and vasculitis from the mouth to the colon (18). Because FK506 and RAP inhibit different target proteins (see below), this strikingly similar toxicity profile in dogs is intriguing. The gastrointestinal toxicity may be due to a chemical property shared by the two compounds or to the fact that both drugs bind to the same intracellular receptors, disrupting an as-yet-undefined FKBP-dependent process in the dog gut. The gastrointestinal side effects are not species-specific, as severe vomiting and intestinal vasculitis are also observed in baboons treated with RAP (26). Another adverse side effect associated with RAP-treatment is testicular atrophy, observed in both mice and cynomolgus monkeys (32). Interestingly, relative to all other human tissues examined, the mRNA encoding the target of the FKBP · RAP complex, mTOR, is very highly expressed in testis (33), suggesting a functional role in testicular physiology.

As studies with FK506 and CsA have shown, toxicity, or the lack thereof, in animals does not necessarily extrapolate to humans (34, 35), and despite its significant adverse side effects, RAP has exhibited sufficient therapeutic potential to proceed to Phase II clinical trials (36–38). Consideration has also been given to the use of RAP in combination with CsA, which may result in additive or even synergistic immunosuppressive effects, with the attendant reduction in individual drug dosage leading to a decreased incidence of adverse side effects (39). The toxicity associated with CsA, FK506, or RAP therapy is not unexpected. The immunophilin complexes containing CsA, FK506, or RAP inhibit the functions of specific but ubiquitously expressed target proteins in mammalian tissues. The profiles of therapeutic and toxic effects observed with each drug likely reflect the abundance and functions of the cognate target proteins, as well as pharmacodynamic parameters such as each drug's ability to penetrate cellular membranes, the concentrations of different immunophilins within various cell types, and the relative affinities of the various immunophilin • drug complexes for their target proteins.

MOLECULAR MECHANISM OF RAPAMYCIN ACTION

The groundwork for understanding the molecular basis of RAP-induced immunosuppression was provided by a remarkable series of mechanistic insights

into the cellular pharmacology of CsA and FK506. CsA and FK506 both inhibit the same subset of Ca^{2+}-associated activation pathways (40), exert their inhibitory effects during the G_0-to-G_1 phase of T cell activation, and block expression of the same set of early lymphokine genes (41). CsA and FK506 bind to abundant, ubiquitous, and phylogenically well-conserved intracellular receptors (42, 43). The major cytosolic receptor for CsA is cyclophilin A (CyPA), an 18-kDa protein (44), while the major FK506 binding protein (FKBP) is a 12-kDa cytosolic protein termed FKBP12 (45, 46). Both CyPA (47, 48) and FKBP12 (45, 46) are enzymes, termed peptidyl-prolyl isomerases (PPIases), that catalyze *cis-trans* isomerization of peptidyl-prolyl bonds in peptides and proteins. FKBP12 prefers substrates with hydrophobic amino acids immediately preceding the proline, while CyPA is a more promiscuous enzyme (49). Although the PPIase activities of CyPA and FKBP12 are inhibited by CsA (47, 48) and FK506 (45, 46), respectively, inhibition of PPIase activity is unrelated to immunosuppression (50–52). These observations suggested a gain-of-function model in which FK506 and CsA are inactive alone but serve as co-drugs with their cognate immunophilins, forming active complexes that inhibit a Ca^{2+}-dependent signal transduction event (53). Subsequently, it was shown that the CyPA · CsA (54) and the FKBP12 · FK506 (55) complexes bind to and inhibit the same target protein, the Ca^{2+}-dependent serine-threonine phosphatase, calcineurin (CaN), a critical component of the TcR-linked signal transduction pathway leading to cytokine gene transcription (56, 57). CaN is activated by the increase in cytoplasmic free Ca^{2+} that results from TcR engagement. A target, either direct or indirect, for the activated phosphatase is the phosphorylated cytoplasmic subunit of the T cell–specific transcription factor, nuclear factor of activated T cells (NFAT). The dephosphorylated cytoplasmic NFAT subunit is free to translocate to the nucleus and associate with a nuclear subunit to form the fully active NFAT complex, an essential component of the transcriptional apparatus required for expression of the IL-2 and other cytokine genes. Thus, by interfering with the TcR-mediated activation of CaN, both CsA and FK506 block transcription of the IL-2 gene in T lymphocytes (for reviews, see 58–60).

The Intracellular Receptors for RAP

RAP and FK506 bind to the same family of intracellular receptors, termed FK506 binding proteins (FKBPs). Structural studies have shown that FK506 has two domains—a domain bound by FKBP and an effector domain that, together with FKBP, forms a composite surface that interacts with CaN (reviewed in 61; 62). Like FK506, RAP also has two domains—an effector domain forming a composite surface with FKBP that interacts with the mammalian target of RAP, mTOR (see below), as well as a binding domain that mediates the

interaction with FKBP. The FKBP-binding domain is conserved in FK506 and RAP, providing a chemical basis for the mutual antagonism exerted by the two molecules in intact cells (16, 63).

The crystal structure of the human FKBP12 · RAP complex has been solved and shows the pipecolinyl ring of RAP buried deeply in the hydrophobic cavity located between the α-helix and β-sheet of FKBP12 (64). The common chemical elements of RAP and FK506 (Figure 1), which include the pipecolinyl ring, C1 ester, pyranose ring, and the C8 and C9 carbonyls, adopt superimposable conformations in the FKBP12 · RAP and FKBP12 · FK506 complexes (64). In contrast to FK506, which undergoes a dramatic conformational change upon binding FKBP12 (65), the three-dimensional structure of RAP complexed to FKBP12 is almost identical to its conformation in the free crystalline state (64). Thus, relative to FK506, RAP is in an energetically favorable conformation for binding to FKBP12, providing an explanation for its two-fold greater affinity.

The human FKBP family is currently comprised of seven members, whose characteristics are summarized in Table 1. Exhaustive reviews on FKBPs can be found elsewhere (66, 67). All FKBPs bind RAP with greater affinity than FK506, with the exception of FKBPr38 (FKBP-related 38-kDa protein) which does not bind the immunosuppressants but is included in the family because of the similarity of its sequence (68). The PPIase activity of all FKBPs is inhibited by RAP and FK506. Because the drug-binding domain overlaps with the PPIase active site, inhibition of PPIase activity by RAP or FK506 can be taken as a measure of the affinity of a particular FKBP for the drug. Among the FKBPs, FKBP25 is most selective for RAP relative to FK506 (69). Despite this selectivity, FKBP25 does not mediate the RAP-sensitivity of mast cells (70), suggesting that the FKBP25 · RAP complex does not interact with mTOR (see below) in intact cells. To date, two FKBP isoforms, FKBP12 and FKBP12.6, have been shown to bind mTOR in the presence of RAP, although the FKBP12 • RAP complex appears to be superior in this regard (71).

There is no evidence linking the physiological function of the FKBPs to their inhibitory functions in the presence of RAP. FKBP12 and FKBP12.6, which associate with mTOR in the presence of RAP, normally interact with the calcium release channel (CRC)/ryanodine receptors (RyR) of the terminal cisternae of skeletal muscle and heart muscle sarcoplasmic reticulum, respectively, and they are required for proper channel function (72–76). The ryanodine receptors in skeletal muscle (isoform 1, RyR-1) and heart muscle (isoform 2, RyR-2) are the largest ion channel complexes known (MR, 2.3×10^6 daltons), and they play an important role in release of Ca^{2+} during excitation-contraction coupling. The skeletal muscle CRC can be represented as (RyR-1 protomer)$_4$(FKBP12)$_4$, while the cardiac muscle CRC can be represented

Table 1 The family of human FKBPs

FKBP	MW (kDa)	% Identity to FKBP12	Affinity (nM) RAP	Affinity (nM) FK-506	PPIase activity[g]	Binds mTOR[h]	Inhibition of CaN[j] (IC_{50})	Physiological association
FKBP12	11.8	100	0.2 (K_d)	0.6 (K_d)	yes	yes	8 nM	RyR-1[k]; IP_3R[l]
FKBP12.6[a]	11.6	83	0.2 (K_d)[d]	0.5 (K_d)	yes	yes	8 nM	RyR-2[m]
FKBP13	13.3	50	ND[e]	55.0 (K_i)	yes	ND	30 μ M	Lumen of ER
FKBP25	25.0	40	0.9 (K_i)	160 (K_i)	yes	ND	$> 50\ \mu M$	Casein kinase II; nucleolin
FKBPr38[n]	38.3	33	no binding	no binding	no	NA[i]	NA[i]	
FKBP51[b]	51.2	50	29 (IC_{50})[f]	166 (IC_{50})[f]	yes	ND	3 μM	
FKBP52[c]	51.8	53	8 (K_i)	10 (K_i)	yes	ND	30 μM	Glucocorticoid receptor; hsp9C

[a]An alternative splice product of the FKBP12.6 mRNA encodes an 8.8 kDa FKBP-related protein (133). [b]This human cDNA has recently been cloned (Baughman G et al, unpublished results). [c]Also known as hsp56, FKBP59, p59, hsp59. [d]Based upon the K_d for FK-506 and adjusted for RAP's greater ability to inhibit PPIase activity (71). [e]Not determined. [f]These values are for murine FKBP51 (134). [g]RAP and FK-506 inhibit PPIase activity in all FKBPs. [h]When the indicated FKBP is complexed with RAP. [i]Not applicable because FKBPr38 (FKBP-related 38 kDa protein) does not bind RAP or FK-506. [j]When the indicated FKBP is complexed with FK-506 (71). [k]Isoform 1 of the ryanodine receptor calcium release channel in skeletal muscle sarcoplasmic reticulum. [l]The inositol triphosphate receptor on the endoplasmic reticulum. [m]Isoform 2 of the ryanodine receptor calcium release channel in cardiac muscle sarcoplasmic reticulum. [n]The 38 kDa mass is based upon translation of the ORF in an isolated cDNA.

as (RyR-2 protomer)$_4$(FKBP12.6)$_4$ (73, 74). Both RAP and FK506 displace FKBP12 from RyR-1 (73), but it is unlikely that at immunosuppressive doses any displacement actually occurs in vivo, since the concentration of FKBP12 in skeletal muscle myoplasm is about 3 μM (74). This would effectively buffer any disruption of the channel complex by either drug. Recently, it has been shown that the IP_3R CRC, a relative of the RyR CRC, is also associated with and modulated by FKBP12 (77). RAP and FK506, but not CsA, can dissociate FKBP12 from the channel, rendering it leaky to Ca^{2+} (77). Again, it is unlikely that immunosuppressive doses of RAP would have an effect on the IP3R CRC in T cells. Only 3–5% FKBP occupancy is required to prevent activation of T cells, and, as in muscle cells, the high intracellular concentration of FKBP (6 to 7 μM) (52) would buffer any effect that RAP might have on the IP_3R-associated FKBP12.

The Targets of the FKBP · RAP Complex in Yeast

Studies in yeast, in which two independent groups first identified the target of the FKBP · RAP complex, have made enormous contributions to our understanding of the mechanism of RAP action in mammalian cells. The extreme growth-sensitivity of *S. cerevisiae* to RAP ($IC_{50} = 0.1$ μg/ml) allowed for selection of RAP-resistant mutants that identified genes mediating RAP sensitivity. Mutant alleles of three genes *FKB1, TOR1 (DRR1),* and *TOR2 (DRR2)* (TOR: target of RAP; DRR: dominant RAP resistant), were found to confer resistance to RAP (78–82). Mutations in *FKB1* (encoding FKBP12) were recessive while the *TOR1* and *TOR2* mutations isolated in these studies were dominant or semi-dominant (80–82). *TOR1*-disruptants exhibit a mild phenotype, growing 10–15% more slowly than wild-type strains (82). When grown in the presence of RAP, the *TOR1*-disruptants arrest growth in early G_1 within one generation. The TOR2 disruption confers a lethal growth defect with cells arresting not only in G_1 but randomly throughout the cell cycle. Strains disrupted for both *TOR1* and *TOR2* arrest growth in G_1 within one generation, mimicking the phenotype of wild-type yeast grown in the presence of RAP. The G_1 arrest phenotype of the *TOR1/TOR2* double-disruptant indicates that RAP inhibits both the TOR1 and TOR2 proteins (82). The presence of either TOR1 or TOR2 is sufficient to allow yeast cells to progress through G_1, indicating that either protein can execute the essential G_1 function. TOR2 has two functions, a RAP-sensitive G_1 function complemented by TOR1 and a RAP-insensitive essential function not complemented by TOR1 (80). The observation that placement of a *TOR2*-disruption in a *TOR1*-mutant, RAP-resistant background confers a lethal phenotype (83) does support the initial suggestion (80) that TOR2 has two functions.

TOR1 and TOR2 are proteins with 2470 (281.2 kDa) and 2474 (282 kDa) amino acid residues, respectively, which lack obvious signal sequences or

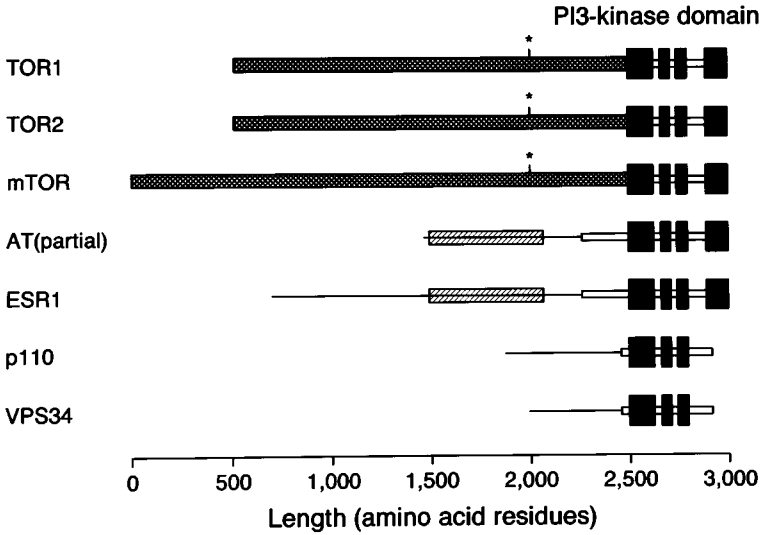

Figure 2 A schematic representation of TOR family members and related proteins. The *asterisk* marks the location of the critical serine residues, Ser^{2035}, Ser^{1972}, and Ser^{1975}, in mTOR, TOR1, and TOR2, respectively. Similarly shaded boxes indicate regions of high homology among PI 3-kinase family members.

transmembrane domains (80, 81). The two proteins have 67% amino acid identity and 80% similarity overall (84). The N-terminal segments of both proteins are unrelated to any protein currently in the databases. The C-terminal regions of TOR1 and TOR2 are homologous to a growing family of signaling proteins. These include (i) the p110 catalytic subunit of bovine phosphatidylinositol 3-kinase (PI 3-kinase), (ii) a PI 3-kinase (VPS34) involved in targeting soluble hydrolases to vacuoles in *S. cerevisiae* (85), (iii) a protein (ESR1, MEC1) required for the repair of damaged DNA and for meiotic recombination in *S. cerevisiae* (86), and (iv) the protein encoded by the human *AT* gene, which is mutated in the autosomal recessive disorder ataxia telangiectasia (87). The regions of greatest homology among the six proteins (amino acids 2123–2296 of TOR1, 2127–2300 of TOR2, 801–935 of p110, 623–751 of VPS34, 2078–2245 of ESR1, and 1368–1543 of the protein encoded by the partial AT cDNA clone) span a putative "lipid kinase motif" containing residues conserved in the ATP-binding domains of PI 3- and PI 4-kinases as well as certain protein kinases (Figure 2).

The lipid kinase domains in TOR1 and TOR2 are functionally interchangeable (82). Thus, the different phenotypes of the TOR1- and TOR2-disrupted yeast reflect functional differences in the amino-terminal regions of each protein.

All TOR1 and TOR2 alleles that have been cloned from RAP-resistant yeast strains are missense mutants that alter the same serine residue just upstream of the lipid kinase domain (80–82, 84). The FKBP · RAP complex binds a 196 amino acid fragment of TOR2 (amino acids 1886–2081), an interaction abolished by mutations of Ser^{1975} (88). Likewise, the binding domain of TOR1 has been mapped to a small region surrounding Ser^{1972}, and mutations to any residue other than alanine prevent binding of FKBP· RAP and confer resistance to RAP (83). Because alanine is a potential mimic of a nonphosphorylated serine residue, these results indicate that phosphorylation of Ser^{1972} is not required for FKBP · RAP binding, and they also argue against an earlier proposal that phosphorylation of $Ser^{1972/1975}$, present in a consensus protein kinase C site, may be required for binding FKBP12 · RAP (82).

Although it has not yet been demonstrated that TOR1 and TOR2 actually possess kinase activities, genetic evidence strongly suggests that intact lipid kinase domains are required for their G_1 function. The introduction of mutations analogous to those known to abolish the lipid kinase activities of VPS34 and mammalian p110 into the lipid kinase domains of the dominant RAP-resistant *TOR1* or *TOR2* alleles abrogates their ability to confer resistance to RAP (83). The reversion to RAP-sensitivity of these strains demonstrates that functional kinase domains in TOR1 and TOR2 are necessary for their G_1 function. When overexpressed in a wild-type background, "kinase dead" *TOR1* mutants confer a dominant negative phenotype, resulting in G_1 arrest (83). This finding suggests that the overproduced, mutant TOR1 protein is nonproductively interacting with normal G_1 targets of the wild-type TOR1 kinase domain. The essential non-G_1 function of TOR2 also requires an intact kinase domain. These genetic data support a model (83) which proposes that the G_1 target of the TOR kinase domains binds to the C-terminus of the TOR proteins, an interaction blocked by FKBP12 · RAP. The model further suggests that the target substrate for TOR2's essential viability function binds to a different region, presumably located in the polymorphic N-terminus of TOR2, and its access to TOR2 is not blocked by FKBP12 · RAP.

Recent studies suggest that the TOR proteins function in a signal transduction pathway that coordinates the availability of essential nutrients with progression through the cell cycle. Even when grown on rich medium, yeast cells depleted of TOR function, either by RAP-treatment or by disruption of both *TOR* genes, display a phenotype similar to that of cells entering G_0 during the starvation response. For example, both nutrient deprivation and treatment with RAP cause a rapid and acute reduction in initiation of translation, an accumulation of glycogen, an increase in vacuole size, a greatly increased transcription of known marker genes for nutrient starvation, and an arrest of yeast cells with 1N

DNA (89). The putative TOR-restriction point has been mapped to a point prior to the α-mating factor arrest point, START (89). TOR-depleted cells arrest in early G_1 because they are unable to synthesize CLN3, a cyclin required for G_1 progression. CLN3 functions, in part, as a transcriptional activator of genes expressed later in G_1, including CLN1, CLN2, ORFD, HCS26, and CLB5, all of which encode known or candidate cyclins. CLN3 expression is regulated by the translational initiation factor eIF-4E. When the CLN3 ORF is fused to eIF-4E-independent 5′ translational regulatory sequences, RAP-induced G_1 arrest is repressed (89). These results indicate that TOR is a signaling molecule required for eIF-4E-dependent translation when nutrient conditions are favorable for G_1-phase progression.

The Mammalian Target of Rapamycin (mTOR)

Five groups, working independently, identified the mammalian protein target of the FKBP12 · RAP complex. Homologous high molecular weight proteins were isolated from various mammalian sources including bovine brain (FKBP-RAP-associated protein, FRAP) (33), rat brain (RAP and FKBP12 target, RAFT; mammalian target of RAP, mTOR) (90, 91), and human lymphocyte (Sirolimus effector protein, SEP; RAP target, RAPT) (92, 93). In keeping with the precedent yeast nomenclature, we refer to the protein as mTOR. Based upon its migration in denaturing gels, the purified mTOR protein has a molecular weight greater than 200 kDa (33, 90–92). Two observations helped to confirm that mTOR mediates the inhibitory effects of RAP in mammalian cells. First, two structural analogs of RAP (16-keto-RAP and 25, 26 iso-RAP) were identified that bind with high affinity to FKBP12 but which are 100-fold less potent inhibitors of G_1 progression in MG-63 osteosarcoma cells. The FKBP12 complexes with 16-keto-RAP and 25, 26 iso-RAP complexes bind mTOR poorly, if at all (33). Second, little or no mTOR is bound by the FKBP12 · RAP complex in extracts prepared from mutant murine T cell (YAC) lines selected for RAP-resistance (91, 94). Extracts prepared from a RAP-sensitive revertant derived from one of the RAP-resistant T cell lines show wild-type levels of mTOR bound to the FKBP12 · RAP complex (91). Thus, sensitivity of T cells to RAP correlates with binding of mTOR to the FKBP12 · RAP complex.

Microsequencing of the purified mTOR protein enabled the cloning of the complete cDNAs encoding both the human (33) and rat homologs (90, 91). The open reading frames encode 2549 amino acid proteins that have calculated molecular weights of 289 kDa. The protein product of the in vitro–translated rat mTOR cDNA binds to the FKBP12 · RAP complex—formal validation that the cloned cDNA encodes a direct ligand for this complex (91). The mRNA of mTOR is ubiquitously expressed in human tissues with the highest levels found in testis and significant expression found in skeletal muscle (33, 93). Both

human and rat mTOR are slightly more similar to yeast TOR2 (46% identity) than to TOR1 (44% identity), with the greatest similarity (65% identity) found in the C-terminal 600 amino acids of the three proteins. This region of mTOR contains the lipid kinase motif (amino acids 2186–2359) found in the yeast TORs. There are other regions, particularly in the amino terminal portions of the proteins, where there is little or no homology between the yeast TORs and mTOR. Thus, although it is unclear whether mTOR is the functional equivalent of yeast TOR1 or TOR2, the high degree of amino acid sequence identity in the lipid kinase domain suggests that these proteins have similar enzymatic activities. To date, in vitro kinase assays have failed to detect lipid kinase activity in mTOR although a serine autophosphorylation activity has been reported (83).

As shown with the yeast TOR proteins (88), only a small portion of mTOR mediates binding to the FKBP12 · RAP complex. In mTOR, the binding domain has been mapped to amino acids 2025–2114 (95), a 90 amino acid region just upstream of the lipid kinase domain containing a critical serine residue (Ser^{2035}) homologous to the $Ser^{1972/1975}$ in the yeast TOR proteins. As in TOR1, mutation of Ser^{2035} to residues other than alanine abrogates binding of mTOR to FKBP12 · RAP (93, 95), indicating that phosphorylation of Ser^{2035} is not required for binding by FKBP12 · RAP and that Ser^{2035} lies in a structurally critical region that is disturbed when replaced by any residue larger than alanine.

BIOCHEMICAL ACTIONS OF RAPAMYCIN IN MAMMALIAN CELLS

Anti-Proliferative Effects of RAP in Lymphoid and Nonlymphoid Cells

RAP is a potent inhibitor of the growth of most hemopoietic and lymphoid cell lines in vitro (17). Maximal growth-inhibitory effects are usually observed at concentrations of the drug equal to or less than 10 nM. It is particularly striking that RAP suppresses the cytokine-driven proliferation of both nontransformed lymphoid cell lines and the continuous growth of several leukemic cell lines, with virtually equal potency. The increase in doubling time of the cells induced by the drug is accompanied by a dramatic increase in the proportion of G_1-phase cells, which is consistent with the model that the FKBP12 · RAP complex interferes specifically with the progression of G_1-phase cells into S phase (96, 97). The growth-inhibitory effect of RAP on lymphoid and other cell types is effectively antagonized by FK506 (16). Conversely, the ability of FK506 to inhibit antigen-dependent IL-2 production is reversed by RAP. The reciprocal antagonism between FK506 and RAP is explained by the competitive interactions of these drugs with a common intracellular receptor FKBP12.

In contrast to the broadly anti-proliferative effects of RAP on bone marrow–derived cells, the efficacy of RAP as an inhibitor of mesenchymal or epithelial cell growth is variable. For example, the serum-dependent growth of Swiss 3T3 mouse fibroblasts is only marginally affected by nanomolar concentrations of RAP (98), whereas RAP profoundly inhibits the proliferation of MG-63 osteosarcoma cells under the same culture conditions (97). The variable response to RAP may reflect physiologic differences in the extent to which redundant signaling pathways compensate for the loss of mTOR function in different cell lineages. Alternatively, certain established cell lines may have acquired abnormalities in cell-cycle regulation that effectively override the putative G_1-phase checkpoint governed by mTOR.

Other Cellular Actions of RAP

Although the immunosuppressive activity of RAP is commonly attributed to its antiproliferative effect on lymphoid cells, this drug affects a number of other cellular functions that may contribute to its ability to suppress immune responses in vivo. Studies performed with the murine T cell lymphoma, YAC-1, have shown that RAP inhibits IL-1-stimulated production of interferon (IFN)-γ as well as IFN-γ-induced expression of Ly6E antigen on the cell surface (94, 99, 100). Both of these actions are antagonized by FK506, suggesting that they are dependent upon the formation of FKBP12 · RAP complexes in YAC-1 cells. Furthermore, YAC-1 somatic mutants selected for resistance to the growth-inhibitory effect of RAP were correspondingly resistant to the suppressive actions of RAP on cytokine-driven production of IFN-γ and expression of Ly6E (94). As mentioned previously, the resistant phenotype of these YAC-1 clones is apparently explained by a mutational event(s) that leads to a decreased affinity of mTOR for the FKBP12 · RAP complex. These results suggest that, in addition to its cell-cycle regulatory function, mTOR participates in the signal transduction pathways that mediate IL-1- and IFNγ-stimulated responses in YAC-1 cells.

Ligation of the CD28 receptor on T cells provides an important costimulatory signal for antigen-dependent cytokine production by helper T-lymphocytes. The signal transduction pathway initiated by the interaction of CD28 with its ligands B7-1 and B7-2 remains obscure. In contrast to signaling from the TCR, signal propagation from the CD28 receptor is not inhibited by CsA or FK506. Conversely, signaling through CD28, but not the TCR, is sensitive to RAP (101). The CD28 response element in the IL-2 promoter region contains a nucleotide sequence that resembles a NFκB-binding site, suggesting that CD28 promotes transcription of the IL-2 gene through the activation of members of the Rel family of transcription factors (102). Stimulation of Jurkat or human peripheral blood T cells with phorbol ester induces the nuclear translocation of the NFκB

family member c-Rel, and both the rate and magnitude of this response are enhanced by costimulation through CD28 (103). The increase in intranuclear c-Rel is accompanied by a sustained downregulation of the inhibitory IκB-α subunit in the cytoplasm. Both responses are inhibited by RAP, but not by CsA, suggesting that the RAP-sensitive target protein, mTOR, is involved in the coupling mechanism between CD28 receptor stimulation and c-Rel-dependent transcription. The impact of RAP on CD28 signaling clearly warrants further investigation, as this drug effect could represent a crucial component of the overall immunosuppressive action of RAP in vivo.

Activation of p70S6 Kinase

The potent antiproliferative effects of RAP prompted speculation that the pharmacologically active immunophilin · drug complex targets a protein kinase involved in the relay of mitogenic signals from the cytoplasm to the nucleus. The most obvious candidate was a component of the Ras-to-MAP-kinase (MAPK) signaling cascade (i.e. Raf, MAPK kinase, or MAPK itself). However, subsequent studies failed to uncover any detectable effect of RAP on Ras signaling in mammalian cells (98; RT Abraham, unpublished data). Instead, the drug was shown to disrupt a Ras-independent signal transduction pathway required for activation of the 70-kDa S6 protein kinase ($p70^{S6K}$). Activation of $p70^{S6K}$ is apparently a universal response of mammalian cells to mitogenic stimuli. In activated T cells, the activity of $p70^{S6K}$ increases within 10 min of addition of IL-2, and activation is maximal after 40–60 min of stimulation (104). This response is accompanied by a decrease in the electrophoretic mobility of $p70^{S6K}$, which likely reflects an increase in the phosphorylation of the enzyme. Pretreatment with RAP abolishes both the shift in electrophoretic mobility and the increase in catalytic activity of $p70^{S6K}$ induced by growth factors in T cells as well as in all other mammalian cell types (98, 104–106). The drug concentrations needed to abolish activation of $p70^{S6K}$ are identical to those required to block the progression of mitogen-stimulated cells through G_1-phase. The suppressive effect of RAP on activity of $p70^{S6K}$ is reversed in the presence of excess FK506, indicating that this action is dependent on the binding of RAP to an FKBP, probably FKBP12. A striking observation is that the addition of RAP to cells at any time after exposure to growth factor leads to a rapid decline in activity of $p70^{S6K}$ to (or even below) the basal level observed in quiescent cells. The ability of RAP to inhibit preactivated $p70^{S6K}$ suggests that the FKBP12 · RAP complex disrupts a constitutive signaling event required for both the generation and maintenance of the activated form of $p70^{S6K}$.

The biochemical results described above are consistent with the idea that the FKBP12 · RAP complex interferes directly or indirectly with the function of a

$p70^{S6K}$-activating protein kinase in mitogen-stimulated cells. As $p70^{S6K}$ itself is not sensitive to RAP (98), the drug's site of action is thought to lie at an upstream point in the signaling pathway that links stimulation of growth factor receptors to activation of $p70^{S6K}$. It is becoming increasingly evident that $p70^{S6K}$ does not lie in a simple, linear signaling cascade, and recent studies suggest that at least two $p70^{S6K}$-activating protein kinases regulate the state of phosphorylation of the enzyme in growth factor-stimulated cells (107, 108). The carboxy terminus of $p70^{S6K}$ contains a 25 amino acid segment whose sequence suggests that it functions as an autoinhibitory pseudosubstrate domain. According to the current model, phosphorylation of the carboxy-terminal regulatory domain at multiple serine and threonine residues relieves an inhibitory constraint on the catalytic domain and allows activation of the enzyme. However, activation of $p70^{S6K}$ also requires an additional stimulatory input mediated through a highly acidic stretch of amino acids in the amino-terminal region (residues 29–46 in the rat protein) of $p70^{S6K}$. Mutational analyses of $p70^{S6K}$ indicate that it is the essential activating signal delivered through the amino-terminal segment that is blocked by RAP (107, 108). Although treatment with RAP has no effect on the mitogen-induced phosphorylation of the carboxy-terminal pseudosubstrate domain, it inhibits phosphorylation elsewhere in $p70^{S6K}$. The simplest interpretation of these results is that the amino-terminal acidic region mediates the interaction of $p70^{S6K}$ kinase with a RAP-sensitive, $p70^{S6K}$-activating kinase. If correct, this model places the RAP target protein, mTOR, in a protein-serine-threonine kinase cascade leading to activation of $p70^{S6K}$.

Does inhibition of $p70^{S6K}$ explain the growth-suppressive action of RAP in lymphoid cells? Unfortunately, a straightforward answer to this question is not yet available. Microinjection of neutralizing anti-$p70^{S6K}$ antibodies into rat embryo fibroblasts blocks serum-induced G_1- to S-phase progression, suggesting that this enzyme plays a key role in transduction of mitogenic signals (109). However, the universality of this conclusion is challenged by experiments with RAP itself. Whereas RAP blocks growth factor-dependent activation of $p70^{S6K}$ with virtually equal potency in both lymphoid and nonlymphoid cells, the efficacy of RAP as a growth inhibitor is relatively unpredictable, particularly in nonhemopoietic cell lines. Thus, if $p70^{S6K}$ executes a critical function required for S-phase entry, redundant signaling pathways must compensate for the loss of $p70^{S6K}$ activity in cells that are relatively insensitive to the growth-inhibitory action of RAP.

Effects of RAP on Protein Synthesis

The progression of mitogen-stimulated cells through G_1 phase is contingent upon regulated alterations in both the transcription of specific genes and the translation of certain mRNA transcripts. Initiation of translation is generally

the rate-limiting step in protein synthesis and therefore represents a logical point of regulation (110, 111). Eukaryotic mRNAs bear a 7-methylguanylate (m^7GTP) cap at the 5′-terminus, and this cap region serves as the recognition site for the eukaryotic initiation factor (eIF)-4F complex. Two components of the multisubunit eIF-4F complex are the ATP-dependent helicase eIF-4A and the mRNA cap-binding protein eIF-4E. Initiation involves the recognition of the m^7GTP cap by eIF-4E, followed by the eIF-4A-dependent unwinding of 5′ secondary structure in the target mRNA. These events are thought to facilitate binding of the methionyl tRNA-charged 40S ribosomal subunit to the mRNA. This model predicts that the degree to which individual mRNA transcripts depend on the eIF-4F complex for initiation of translation varies directly with the complexity of the secondary structure at the 5′-terminus of the mRNA. Thus, the translation of particular mRNAs will be variably sensitive to regulatory alterations in the function of eIF-4F. As might be expected, a major mechanism by which eIF-4F is regulated involves the phosphorylation of specific components of the eIF-4F complex.

An important target of protein kinases involved in controlling initiation of translation is the cap-binding protein eIF-4E. Recent studies have shown that the binding activity of eIF-4E is regulated by its interaction with PHAS-I, a heat-stable inhibitor of eIF-4E function (112, 113). In resting cells, PHAS-I is tightly bound to eIF-4E and thereby inhibits the ability of eIF-4E to trigger eIF-4F-dependent initiation. Stimulation of cells with growth factors, including serum, insulin, or IL-2, leads to the hyperphosphorylation of PHAS-I, and release of its associated eIF-4E. Hence, mitogenic stimuli are capable of stimulating rapid increases in initiation of translation by activating protein kinases that phosphorylate PHAS-I. Although PHAS-I is an excellent in vitro substrate for MAP kinase, the crucial phosphorylation events leading to the release of eIF-4E are apparently not performed by this enzyme in intact cells (114). Rather, the PHAS-I kinase responsible for disinhibition of eIF-4E in vivo lies within a RAP-sensitive signaling pathway. Pretreatment of activated T cells or 3T3-L1 fibroblasts with RAP abolishes both the increase in phosphorylation of PHAS-I and the release of active eIF-4E induced by IL-2 or insulin (114; GJ Brunn, RT Abraham, J Lawrence, unpublished observations). These results strongly suggest that an mTOR-regulated protein kinase controls eIF-4E-dependent initiation of translation in mammalian cells. These findings are similar to those found in yeast. Thus, studies with RAP as a probe have uncovered a general mechanism for the control of protein synthesis in eukaryotic cells.

The inhibitory effects of RAP on phosphorylation of PHAS-I and the function of eIF-4E provide a rational explanation for the selective, rather than global, suppressive actions of RAP on mitogen-induced protein synthesis. For example,

RAP specifically interferes with the synthesis of proteins from a class of mRNAs bearing polypyrimidine tracts at their 5′-termini (115, 116). Included among these polypyrimidine-containing mRNAs are transcripts that encode ribosomal proteins and elongation factors, i.e. components of the protein synthetic machinery itself. In many cases, the 5′-untranslated regions of these mRNAs also contain nucleotide sequences predicted to have considerable secondary structure, thereby making initiation of translation strongly dependent on eIF-4E. Therefore, the translation of this class of mRNAs should be particularly sensitive to RAP.

In summary, accumulating evidence suggests that the growth-inhibitory mechanism of RAP is inextricably linked to the machinery that controls the inducible synthesis of specific proteins in growth factor–stimulated cells. If this model is correct, the next task will be to identify the mRNA transcript(s) whose translation limits the rate of passage of growth factor-stimulated lymphoid cells through the mid/late G_1-phase checkpoint defined by RAP. This effort will be greatly facilitated by an understanding of the effect of RAP on the biochemical machinery that controls the passage of cycling cells through G_1 and into S phase: the G_1 cyclins and their associated cyclin-dependent kinases (cdks).

Effect of RAP on G_1 Cyclin-cdk Activities

The progression of growth factor-stimulated mammalian cells from G_1 to M phase is orchestrated by the precisely timed activation and inactivation of a series of cyclin-associated protein kinase activities (see 117–121 for reviews). The sequence and timing of activation of each cyclin-cdk complex is controlled in part by cell-cycle checkpoints, which ensure that the appropriate cyclin-cdk complex becomes active only after contingent earlier events are accurately executed (119). The G_1-specific growth-arrest state induced by RAP suggested that this drug might interfere, directly or indirectly, with the timely activation of a G_1 cyclin-cdk complex required for the entry of IL-2-stimulated, G_1-phase T cells into S phase. Progression through G_1 phase is marked by the assembly and catalytic activation of at least three sets of cyclin-cdk complexes. In IL-2-stimulated T cells, the first set of complexes to become active reflects the association of cyclin D2 with cdk4 or cdk6. This event is followed by the sequential activation of cyclin E-cdk2 and cyclin A-cdk2 complexes (116, 117). It is believed that the first two sets of cdk complexes containing cyclins D2 and E set up the conditions for passage of the cell through a restriction point in late G_1-phase (122). Thereafter, the cell is fully committed to complete one division cycle. In contrast, cyclin A-cdk2 activity, which begins to rise in late G_1 and remains elevated throughout S-phase, performs functions needed for the onset and completion of replication of DNA.

Exposure of activated T cells to RAP profoundly affects the appearance and/or functions of the G_1 cyclin-cdk activities induced by IL-2. Surprisingly little has been reported concerning the effect of RAP on the cyclin D–associated cdk complexes in this cell type. Preliminary studies suggest that RAP strongly interferes with the activation of cdks complexed with either cyclin D2 or cyclin D1 in YAC-1 T lymphoma cells or MG-63 osteosarcoma cells (97; GJ Brunn, RT Abraham, unpublished data). Conceptually more interesting results were obtained from biochemical analyses of the cyclin E-cdk2 complexes in IL-2-responsive T cells treated with RAP (123). The drug-treated cells express near-normal levels of cyclin E, and this cyclin is stoichiometrically associated with cdk2. However, the cyclin-E-cdk2 complexes assembled in the presence of RAP display essentially no detectable protein kinase activity. Although additional phosphorylation and dephosphorylation events are required to activate cyclin E-bound cdk2, the phosphorylation state of cdk2 indicated this protein kinase should be fully active in cells growth-arrested by RAP. The mechanism underlying the inhibitory effect of RAP on cyclin E-cdk2 activity remained elusive until the discovery of a novel family of negative regulators of the cell cycle termed cdk inhibitors (124).

One member of this family is Kip1, a heat-stable, titratable inhibitor of G_1 cyclin-cdk activities (125–128). Because Kip1 inhibits these activities in a stoichiometric rather than a catalytic fashion, the level of expression of Kip1 sets a threshold on the numbers of G_1 cyclin-cdk2 complexes that must be assembled before the formation of catalytically active complexes can occur (Figure 3). Recent studies using human peripheral blood T cells have shown that the initial activation step results in the expression of all three G_1 cyclins (D2, E, and, to a lesser extent, A) together with their cdk partners (cdk4, cdk6, and cdk2) (129). However, these complexes fail to become active until the cell receives a progression signal provided by IL-2 or other growth-promoting cytokines. The block to G_1 progression is explained, at least in part, by the presence of very high levels of Kip1 in activated T cells. Stimulation with IL-2 overcomes this Kip1-imposed block by inducing both a progressive decrease in the level of Kip1 protein and the assembly of additional G_1 cyclin-cdk complexes (129, 130). A provocative addendum to this regulatory scheme was provided by the recent finding that the degradation of Kip1 observed in mitogen-stimulated mammalian cells is carried out by the ubiquitin-proteasome pathway (131). This result adds Kip1 to the growing list of cell cycle–related proteins whose functions during each cycle are terminated by their timed destruction via ubiquitin-triggered proteolysis.

RAP tips the balance between activation and inhibition of cdks toward the inhibitory side by blocking the downregulation of Kip1 protein normally

provoked by IL-2 (150). Under these circumstances, the available G_1 cyclin-cdk complexes remain saturated with Kip1 and are unable to execute critical regulating functions, including the phosphorylation of the retinoblastoma protein (132), that are essential for progression through G_1. The persistent expression of Kip1 in RAP-treated T cells suggests that mTOR, the target for the FKBP12 · RAP complex, is a critical component of a signaling pathway that marks the Kip1 protein for ubiquitin-dependent proteolysis. In the light of the earlier discussion, the irony in this mechanism is obvious: mTOR apparently functions as a positive regulator during mitogen-induced protein synthesis, yet the most proximal connection to the cell cycle occurs at the level of the proteolytic destruction of Kip1. Although any model must be considered purely speculative at present, the mTOR-dependent signaling pathway may control the translation of a mRNA species whose protein product allows the ubiquitin-proteosome system to recognize and degrade Kip1. Whatever the actual mechanism, further studies with RAP as a pharmacologic probe are likely to provide some fascinating insights into the mechanism whereby occupancy of growth factor receptors at the cell surface triggers the activation of G_1 cyclin-cdk complexes in the T cell nucleus.

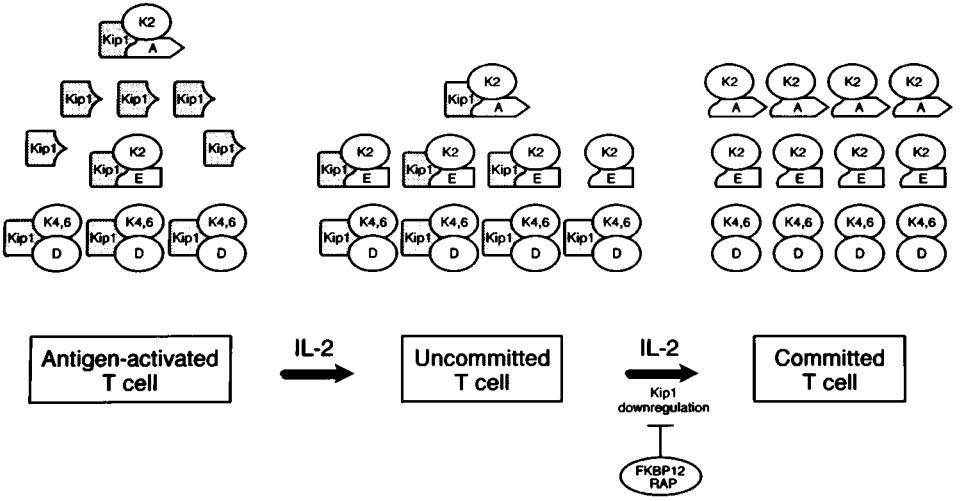

Figure 3 Role of the cdk inhibitor Kip1 in the antiproliferative mechanism of action of RAP. Kip1 functions as a stoichiometric inhibitor of G_1-cyclin-cdk complexes in activated T cells. Cyclin subunits are designated as D, E, and A, and the catalytic cdk subunits are abbreviated as K followed by the number of the appropriate cdk isoform. According to this model, RAP inhibits G_1-phase progression by blocking IL-2-dependent Kip1 downregulation.

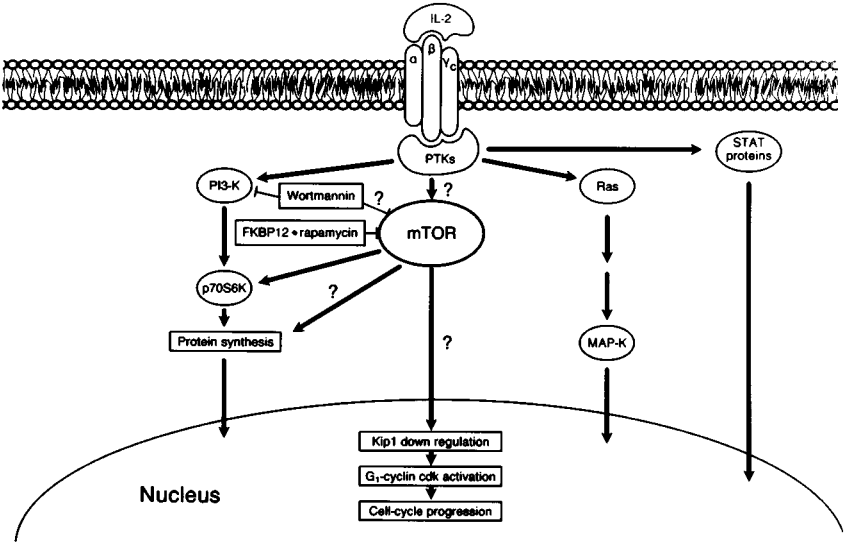

Figure 4 Schematic representation of IL-2 receptor-coupled signaling pathways. The model proposes that mTOR functions as an inducible transducer of regulatory signals for $p70^{S6K}$ activation, eukaryotic initiation factor 4E-dependent protein synthesis, and Kip1 downregulation. Interaction with FKBP12 · RAP disrupts mTOR-dependent functions. Wortmannin, an irreversible inhibitor of certain PI 3-kinase family members, also interferes with a subset of mTOR-dependent responses, including $p70^{S6K}$ activation.

CONCLUDING REMARKS

Studies of the mechanism of action of RAP are beginning to uncover a previously unrecognized signal transduction pathway that may play a general role in the control of growth of hematopoietic cells (see Figure 4 for summary). The FKBP12 · RAP complex binds to and inhibits the function of a newly defined target protein termed mTOR. The remarkable degree of sequence identity between mTOR and its yeast homologs TOR1 and TOR2 suggests that the cell-cycle regulatory function of mTOR has been highly conserved in eukaryotes. Sequence similarities in the putative catalytic domain of mTOR indicate that the RAP target protein is evolutionarily related to the phosphoinositide kinases PI 3-kinase and PI 4-kinase. The most recent addition to this expanding family of signal transducers is the product of the human *AT* gene, which is mutated in the autosomal recessive disorder ataxia telangiectasia (87). Mutations in AT result in neurologic, immunologic, and cell-cycle abnormalities, as well as radiation-sensitivity and a predisposition to cancer. These findings hint that members

of the family of PI 3-kinase-like enzymes, including mTOR, are involved in a broad range of physiologic processes linked to control of the cell-cycle.

The growth-arrest state induced by RAP in T lymphocytes and other hematopoietic cells suggests that this protein executes a biochemical function(s) required for progression from G_1- to S-phase. In spite of the sequence homology to PI 3-kinase, mTOR possesses no detectable kinase activity toward phosphoinositides or other lipid substrates (83). However, recombinant mTOR phosphorylates itself on serine residues (83), suggesting that mTOR, ATM, and related proteins may constitute a novel family of protein serine-threonine kinases. Although the proximate substrate(s) for mTOR remains obscure, the connection to components of the machinery that control translation of mRNA is becoming increasingly compelling. The next few years should see some exciting advances in our understanding of the functions of mTOR and other members of this protein family. It is anticipated that these advances will seed the development of novel strategies for immunosuppression, and for the treatment of leukemias, lymphomas, and other cancers.

NOTE ADDED IN PROOF

While this review was being prepared for publication, Brown et al (135) reported findings which strongly support the model that mTOR represents the RAP-sensitive regulator of $p70^{S6K}$ in mammalian cells. Both the putative catalytic domain at the carboxy terminus and the amino terminal domain of mTOR were required for the regulatory action of this protein on $p70^{S6K}$ activity in vivo. Furthermore, this study shows that recombinant mTOR autophosphorylates in vitro and that this autokinase activity is sensitive to inhibition by the FKBP12 • RAP complex.

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RECOGNITION BY γ/δ T CELLS

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ABSTRACT

In contrast with the study of $\alpha\beta$ T cells, that of $\gamma\delta$ T cells is relatively recent and stems from the discovery of their rearranged genes, rather than from any knowledge of their biological function. Thus, experiments designed to characterize their specificity and function have drawn heavily on our knowledge of $\alpha\beta$ T cells. During the past few years, many studies, especially with mice lacking either $\alpha\beta$ or $\gamma\delta$ T cells, have demonstrated that $\gamma\delta$ T cells can contribute to immune competence, but they do so in a way that is distinct from $\alpha\beta$ T cells. It is also evident that $\gamma\delta$ T cells may not recognize antigen the same way as do $\alpha\beta$ T cells. Analysis of three protein antigens—the murine MHC class II IE^k, the nonclassical MHC T10/T22, and the Herpes virus glycoprotein gI—indicates that $\gamma\delta$ T cell recognition does not require antigen processing and that the proteins are recognized directly. In all three cases, recognition by these T cell clones involves neither peptides bound to these proteins nor peptides derived from them. Moreover, a group of small phosphate-containing nonpeptide compounds derived from mycobacterial extracts has been found to stimulate a major population of human peripheral $\gamma\delta$ T cells in a T cell receptor (TCR)-dependent manner. This indicates that $\gamma\delta$ T cells can respond to ligands that are different from those of $\alpha\beta$ T cells.

Analysis of complementarity determining region (CDR3) length distributions of γ and δ chains indicates that they are more similar to those of immunoglobulins than to TCR α and β. This further supports the idea that $\gamma\delta$ and $\alpha\beta$ T cells recognize antigens differently and suggests that $\gamma\delta$ T cells may be more like immunoglobulins in their recognition properties. $\gamma\delta$ T cells share many cell surface proteins with $\alpha\beta$ T cells and are able to secrete lymphokines and express cytolytic activities in response to antigenic stimulation. These, together with the results cited above, indicate that $\gamma\delta$ T cells can mediate cellular immune functions without a requirement for antigen processing. Thus, pathogens, damaged tissues, or even B and T cells can be recognized directly, and cellular immune responses

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can be initiated without a requirement for antigen degradation or specialized antigen-presenting cells. This would give $\gamma\delta$ T cells greater flexibility than the more classical type of $\alpha\beta$ T cell–mediated cellular immunity.

1. INTRODUCTION

It has been just over a decade since the accidental discovery of a T cell receptor (TCR) γ chain gene during the search for the TCR α gene (1). This work led to the identification of a novel subset of lymphocytes bearing a new TCR heterodimer, $\gamma\delta$ (2–4). The identification and characterization of the TCR δ gene were led by its location within the TCR α locus (5). While much is known about TCR γ and δ gene organization, sequence repertoire, and ontogeny, our understanding of the role of these cells in the immune system is still limited. Nevertheless, experimental evidence has been accumulating that $\gamma\delta$ T cells do contribute to the immune defense, but in a way that is distinct from $\alpha\beta$ T cells. Although $\gamma\delta$ T cells have been proposed to play a first line of defense and/or sensor role (6), this is at present a working hypothesis, and the exact targets and effects of $\gamma\delta$ cells are now being investigated. Toward this end, major progress has been made in the last few years. Observations from a number of laboratories indicate that $\gamma\delta$ T cells are profoundly different from $\alpha\beta$ T cells in their recognition properties and may even recognize a completely different set of antigens. As several recent comprehensive reviews on $\gamma\delta$ T cell specificity have summarized earlier work (6, 7), this review focuses on new developments pertaining to their recognition requirements and the new opportunities that these insights give us for understanding the function of this enigmatic arm of the immune system.

2. $\gamma\delta$ T CELLS CONTRIBUTE TO THE IMMUNE DEFENSE DIFFERENTLY THAN DO $\alpha\beta$ T CELLS

Many studies aimed at distinguishing the contributions of $\alpha\beta$ versus $\gamma\delta$ T cells to the immune defense have taken advantage of mice deficient for $\alpha\beta$ or $\gamma\delta$ T cells. This has been achieved either by administration in vivo of mAb against $\alpha\beta$ or $\gamma\delta$ T lymphocytes or by disruption of a TCR gene through homologous recombination, resulting in mutant mice devoid of T cells expressing a particular type of TCR. Progression of various infections in terms of survival rate, remission time, kinetics of bacterial growth, and development of specific lesions could thus be followed, and the respective effects of $\alpha\beta$ and $\gamma\delta$ T cell populations appraised (8, 9; RL O'Brien, CE Roark, YX Fu, W Born, in press).

The most thoroughly studied infectious disease model in this setting is that of *Listeria monocytogenes*, an intracellular bacterium (10–12, 16; RL O'Brien,

CE Roark, YX Fu, W Born, in press). Analyses have also been performed with *Leishmania* (13), *Mycobacterium* (12), *Plasmodium* (14), and *Salmonella* (15). Lack of both $\alpha\beta$ and $\gamma\delta$ T lymphocytes resulted in all cases in greater severity of the disease, while the absence of either $\alpha\beta$ or $\gamma\delta$ T cells ameliorated the process somewhat. In most cases, $\gamma\delta$-deficient mice did better than $\alpha\beta$-deficient mice, but the former were still immunocompromised when compared to heterozygotes or normal litter mates (10–12, 14, 16; RL O'Brien, CE Roark, YX Fu, W Born, in press).

All of the published studies indicate that in most cases $\gamma\delta$ T cells can mediate a protective immune response in the absence of $\alpha\beta$ T lymphocytes. Specific effects for $\alpha\beta$ vs $\gamma\delta$ T lymphocytes can also be found, thereby pointing to distinct antigen recognition and/or effector functions of these cell populations. At the same time, at least in some cases, a small degree of compensation seems to exist, illustrating a plasticity in the immune response (12; R O'Brien, CE Roark, YX Fu, W Born, in press).

Beside the general quantitative changes (as reflected in the lethal dose of pathogen), more specific qualitative effects of $\gamma\delta$ T cells have been observed. In some cases, $\gamma\delta$ T cells are responsible for reduced bacterial growth in the early stages of infection (10), whereas $\alpha\beta$ T cells clear bacteria in the later stages (10, 12). This finding is supported by studies with normal mice in which an increase of $\gamma\delta$ T lymphocytes has been demonstrated early in infections by bacteria or a virulent Sendai virus strain, before a response by $\alpha\beta$ T cells was observed (17, 18). This chronology of events also fits with the role as "first line of defense" that has been suggested for $\gamma\delta$ T cells. In contrast, $\gamma\delta$ TCR mRNA$^+$ cells are present within inflammatory lesions late rather than early in the course of infections by influenza or a less virulent Sendai virus (19, 20). It is therefore possible that the functional role of $\gamma\delta$ T cells may differ depending on the host-parasite system. Qualitative differences in the effect of $\alpha\beta$ vs $\gamma\delta$ T cell populations in the later stages of an infection can also be demonstrated by analyzing specific lesions. During infection with *Leishmania*, the absence of $\gamma\delta$ cells results in larger cutaneous lesions compared with those in the absence of $\alpha\beta$ T cells (13). Similarly, larger lesions with abscess formation occur during listeriosis in the absence of $\gamma\delta$ but not of $\alpha\beta$ T cells (11, 12). Vaccine-induced protection is reported to be independent of $\gamma\delta$ cells in listeriosis (12). However, immunization against the malaria parasite *Plasmodium* is effective in $\alpha\beta$-deficient mice, and a $\gamma\delta$ T cell clone can protect normal mice from development of the liver stages of *Plasmodium* (14).

In addition to these differences observed for $\alpha\beta$ vs $\gamma\delta$ T cells in response to immunological challenges, several recent studies also suggest that certain $\gamma\delta$ T cells may have unique functions not directly related to antigen recognition, such

as producing a tissue-specific growth factor (21), regulating the development of epithelial cells (22), controlling $\alpha\beta$ T cell responses (23, 24), and promoting isotype switching in B lymphocytes (25, 26). Anti-tumor effects of a TCRγ transgene have also been reported. In these experiments, the presence of the TCR Vγ1.1 Jγ4 Cγ4 transgene in mice could confer resistance to spontaneous acute T cell leukemias (27). This resistance is influenced by the murine *Nramp* gene. This gene was shown to govern resistance or susceptibility to tuberculosis, leprosis, and leishmaniasis (27a). While no detailed analysis of $\gamma\delta$ T cell specificities has been made, this result raises an additional interesting possible function for these T cells.

Clearly the work on the issue of $\gamma\delta$ T cell function has just begun to bear fruit. Although there are many tantalizing leads, no general statement can be made about the precise nature of the immune deficiency caused by the lack of T cells or about their overall roles. Perhaps the answer will come from further studies in these models, along with a better understanding of antigen recognition (see next section). The function of $\gamma\delta$ T cells has so far been studied extensively only in mouse and human, yet $\gamma\delta$ cells are significantly more abundant in birds (28) and artiodactyls (29). In sheep the $\gamma\delta$ TCRs show an expanded repertoire (30). Thus, the role of $\gamma\delta$ T cells in these species may need to be addressed differently.

3. $\gamma\delta$ AND $\alpha\beta$ T CELLS HAVE DIFFERENT ANTIGEN RECOGNITION REQUIREMENTS

During the past two years, a number of studies have shown that $\gamma\delta$ T cells, as compared with $\alpha\beta$ T cells, have profound differences in their antigen recognition requirements. Some seem to recognize entirely different types of antigens. More specifically, these experiments suggest that there are no antigen-processing and presentation requirements for recognition by most $\gamma\delta$ T cells (31–33), and that their antigens do not have to be proteins (34–37). These experiments are discussed in detail below.

3.1 *Protein Antigens Recognized Directly Without Antigen Processing*

Because most $\alpha\beta$ T cells recognize protein antigens processed inside the cell and presented by MHC molecules, it has often been assumed that $\gamma\delta$ T cells follow the same general pattern. Even when early work showed that classical MHC molecules were not involved in antigen presentation to $\gamma\delta$ T cells, it was assumed that nonclassical MHC molecules, heat shock proteins, or as yet to be identified surface proteins might play a similar role.

Recently, the recognition requirements for $\gamma\delta$ T cells were evaluated in three model systems that have allowed a precise interpretation of the results:

1. Recognition of the mouse class II MHC molecule IE^k by the T cell clone LBK5 (31),

2. recognition of the mouse nonclassical MHC class I molecules T10 and T22 by the T cell clone G8 (31, 33), and

3. recognition of a herpes simplex-virus glycoprotein, gIg by the T cell clone TgI4.4 (32).

The IE^k encoded protein binds peptides, and the T10/T22 molecule has been postulated to do so by virtue of the fact that it is homologous to classical MHC class I molecules. Furthermore, all three proteins listed above can be degraded into peptides and "presented" for recognition. Strikingly, in all three cases, neither peptides bound to these proteins nor peptides derived from them are recognized by the $\gamma\delta$ T cell clones. Instead, protein antigens are recognized directly without any requirement for antigen processing.

3.1.1 IE^K RECOGNITION BY LBK5 IE^k is a classical MHC class II molecule that binds a large number of different peptides as well as most superantigens, and in either capacity this molecule can stimulate $\alpha\beta$ T cells. Analysis of IE^k recognition by the $\gamma\delta$ T cell clone LBK5 allows a comparison of the role MHC molecules play in $\gamma\delta$ vs $\alpha\beta$ T cell recognition; that is, to what extent do peptides bound to MHC confer specificity, what kind of antigen processing is required, and are the MHC molecules recognized similarly in both situations?

The results are very striking. Schild et al (31) found that peptides bound to IE^k do not confer specificity and that no conventional antigen-processing pathways are required when IE^k is recognized by LBK5. As shown in Table 1 and Figure 1*a*, LBK5 recognition does not require a specific peptide MHC complex formed in the endosomal compartments. All variations in the ability of different stimulator cells to activate LBK5 could be attributed solely to the level of their surface MHC expression, regardless of the stimulator cell's species origin (mouse, hamster, human) and cell type (B cells, T cells, fibroblasts). Manipulations that influence the repertoire of peptides loaded onto MHC molecules also showed no effect (GPI-IE^k vs native IE^k, IE^k expressed with or without invariant chains, in the presence or absence of functional class I or II antigen-processing pathways). Likewise, binding particular peptides to the IE molecules did not affect their ability to stimulate LBK5. Isolated IE^k protein bound to a plastic plate could stimulate LBK5 to an extent similar to that of cells that express IE^k (Figure 1*b*), indicating that IE is not presenting a superantigen.

Table 1 Summary of the T cell responses by IE^k on temperature sensitive CHO cell mutants[a]

	2B4 peptide ($\alpha\beta$)		2B4 protein ($\alpha\beta$)		LBK5 ($\gamma\delta$)	
Temp (°C)	34	39	34	39	34	39
IE^k-CHO	+++	++++	+++	++++	++	+++
IE^k-G8.1 End1	+++	++++	++	0	++	+++
IE^k-25.2.2 End2	+++	++++	+++	+	++	+++
IE^k-G7.1 End3	+++	++++	++	0	++	+++

[a]The responses of 2B4 (an $\alpha\beta$ T cell specific for IE^k and moth cytochrome c) and LBK5 to IE^k expressed on three temperature sensitive CHO mutant cells with defects in endosomal acidification induced at 39°C (nonpermissive temperature) are compared with responses elicited from IE^k expressed on normal CHO cells. Each "+" indicates a 5-fold difference in IL-2 or IL-3 production, while "0" indicates no detectable IL-2/IL-3 production. LBK5, but not the 2B4 hybridoma, can be stimulated by all three mutants at 39°C, indicating that LBK5 stimulation is independent of the endosomal pathway utilized by class II MHC molecules. The ability of 2B4 to respond to peptide-loaded mutant cells at 39°C demonstrates that surface expression of transfected IE^k is intact (31).

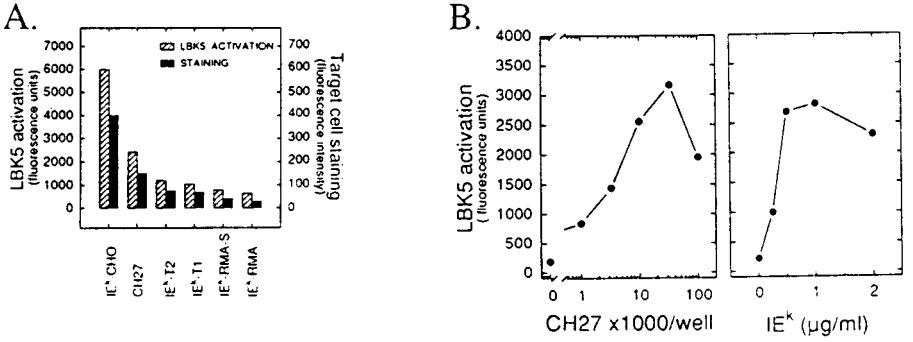

Figure 1 (*a*) Stimulation of LBK5 does not require classical antigen-processing pathways and correlates with surface IE^k expression. LBK5 cells can be stimulated by IE^k–transfected RMA-S and T2, which are defective in class I– and class II–restricted antigen processing, and by their respective parental, nondefective lines RMA and T1. Stimulation of LBK5 correlates directly with the degree of surface expression of the IE^k ligand. In this experiment, LBK5 cells expressing an alkaline phosphatase gene under the control of an IL-2-enhancer promoter were used, and fluorescence units represent measurements of specific alkaline phosphatase activity. The mean fluorescence intensity represents the amount of surface IE^k expression as measured by FACS staining (31). (*b*) LBK5 can be stimulated by plate bound IE^k isolated from CH27 cells. Stimulation of LBK5 by CH27, a B cell lymphoma that expresses IE^k, and by IE^k, isolated from CH27 bound on microtiter wells, is shown. LBK5 stimulations by both were performed in the same assay (31).

In addition, epitope mapping with mutant IE molecules showed that amino acid residues in the α-helices of the IEα and IEβ chains that affect $\alpha\beta$ T cell recognition do not affect LBK5 stimulation (Figure 2). Instead, a mutation at position 79 of the IEα chain (α79) abolished LBK5 recognition but did not affect any of the over 40 $\alpha\beta$ T cells tested. This result indicates that the topology of $\alpha\beta$ vs $\gamma\delta$ T cell recognition of the same IE molecule is likely to be quite different. Further analysis indicated that the specificity of LBK5 is influenced by the carbohydrate at α82 as well as a polymorphic residue at β67, a residue that is exposed to solvent (J Hampl, Y-H Chien, unpublished result).

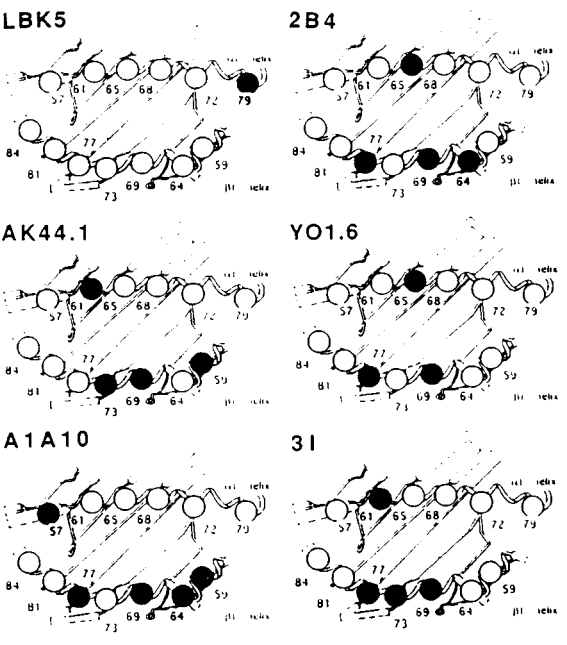

Figure 2 IE^k mutants and their effects on T cell stimulation. The location of mutated residues in IE^k is depicted according to the model of Brown et al (75). The positions and amino acid substitutions of the 13 IE^k mutants are: α1 helix mutants: 57 Ser$\longrightarrow$Asp, 61 Gln$\longrightarrow$Arg, 65 Ala$\longrightarrow$Val, 68 Ala$\longrightarrow$Val, 72 Ala$\longrightarrow$Val, 79 Glu$\longrightarrow$Lys. β1 helix mutants: 59 Glu$\longrightarrow$Lys, 64 Gln$\longrightarrow$Arg, 69 Glu$\longrightarrow$Lys, 73 Ala$\longrightarrow$Val, 77 Thr$\longrightarrow$Gln, 81 His$\longrightarrow$Tyr, 84 Glu$\longrightarrow$Lys. Mutations at positions that showed more than a 1000-fold decrease in presentation efficiency to $\alpha\beta$ T cells are shown in filled circles. For LBK5, only the α79 mutation elicited no response. The dose response curves of LBK5 to all the other mutants are indistinguishable from the LBK5 response to native IE^k. All stimulations were performed with transfected CHO cells. The $\alpha\beta$ T cell hybridomas 2B4, 3I, and YO1.6 are specific for IE^k plus moth cytochrome c, lambda repressor, and hemoglobin peptides, respectively. A1A10 and AK44.1 are both alloreactive for IE^k (31).

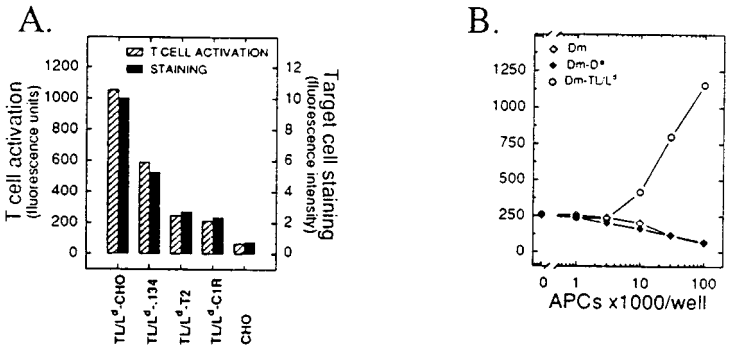

Figure 3 (*a*) Correlation of surface T10/L^d expression on stimulator cells with G8 responses. T10/L^d is a chimera of the $\alpha 1$ and $\alpha 2$ domains of T10 and the $\alpha 3$ domain of the classical class I MHC L^d. Using the monoclonal Ab 28-14-8, specific for the $\alpha 3$ domain of L^d, T10 cell surface expression may be detected and quantified. T10/L^d can stimulate G8 to the same extent as do T10 transfected cells. Stimulation of G8 by T10/L^d expressed on 721.134 and T2 cells, which are defective in antigen processing, illustrates the independence of G8's response from functional antigen-processing pathways. Stimulation of G8 correlates directly with the degree of surface expression of the T10 ligand. G8 cells expressing an alkaline phosphatase gene under the control of an IL-2-enhancer promoter were used, and fluorescence units represent measurements of specific alkaline phosphatase activity. The mean fluorescence intensity represents the amount of surface T10/L^d expression as measured by FACS staining (31). (*b*) Stimulation of G8 by T10/L^d does not require classical antigen-processing pathways. When expressed on Schneider's Drosophila cells, which lack any conventional antigen-processing pathways, T10/L^d can still stimulate G8 effectively. Stimulation assays with mouse class I D^b expressed on Drosophila cells and with untransfected Drosophila cells are shown as controls (31).

3.1.2 T10/T22 RECOGNITION BY G8 The ligands for the T cell clone G8 were recently identified to be the product of the nonclassical class I T10 and the closely related T22 gene (94% identity) (31, 33). As in the case of LBK5 described above, no conventional antigen-processing pathways were required for T10/T22 recognition by the G8 clone. Again all variations in the ability of different stimulator cells to activate these $\gamma\delta$ T cells can be attributed solely to the level of their surface MHC expression, and class I or class II antigen-processing pathways had no effect (Figure 3*a*). Also significant is the fact that G8 recognized T10/T22 molecules expressed on Drosophila cells. *Drosophila melanogaster* does not have an immune system equivalent to that of mammals and appears to lack the factors necessary for any type of antigen processing and presentation (Figure 3*b*).

The T22 molecule has also been identified as the ligand of another $\gamma\delta$ T cell clone, KN6 (38). It is not clear if KN6 also recognizes T10, because the T10 gene assayed by Ito et al (39) is not functional. In a way similar to the results

shown for G8, KN6 responded to T22 expressing cells from a variety of tissues as well as to T22-expressing cells deficient in the class I peptide transporter (TAP1/TAP2). All of these experiments show that conventional peptide loading mechanisms for T cell recognition of classical MHC class I molecules are not necessary for either G8 or KN6 stimulation by the T10/22 gene product.

The T10/T22 sequences have a 3-amino-acid deletion in the predicted $\alpha 1$ helix and a 13-amino-acid deletion in the $\alpha 2$ helix when compared with MHC class I sequences. The amino acid residues following the 13-amino-acid deletion also do not have a helical propensity like that seen in classical MHC molecules. Therefore, even if these molecules fold in a way similar to classical MHC molecules, the α helix would be severely truncated on one side and the β strand platform would be shortened (39). In addition, T10 and T22 lack four of the eight amino acids important for peptide binding by class I molecules (40).

In an effort to evaluate the "peptide" requirements for KN6 recognition, the T22 gene was analyzed by mutagenesis (41). While some residues located on the putative peptide binding floor showed no effect, others reduced KN6 reactivity drastically, leading to the conclusion that a peptide is involved in the recognition. However, in these experiments the cell surface expression of the transfected gene was not monitored, and thus an alternative explanation is that these mutations prevented the proper expression of the protein. In fact, one of those mutations (residue 25 Val$\longrightarrow$Phe) was generated independently and resulted in the loss of surface expression (N Mavaddat, M Crowley, Y-H Chien, unpublished results).

The addition of peptide libraries (7–9 amino acids long, or shorter) does not augment the expression of T10/L^d on the surface of cells defective in the conventional antigen-processing pathway (H Schild, M Jackson, Y-H Chien, unpublished results); neither could peptide be eluted from T10/L^d using standard methods (A Kaliyaperumal, R Falchetto, A Cox, R Dick, Y-H Chien, L Matis, DF Hunt, JA Bluestone, in press). In addition, we have recently found that T10 and $\beta 2$ microglobulin expressed in *E. coli* are able to fold into stable complexes without exogenously added peptide. The folded material bound onto plastic plates can stimulate G8 to an extent similar to that of cells expressing T10 (M Crowley, N Mavaddat, Y-H Chien, manuscript in preparation). Thus, not only does G8 recognize T10 in the absence of peptide, but it also seems that T10/T22 does not bind peptide. At least one of the MHC-like molecules, the FcRn receptor, has a closed groove and thus is unable to bind peptides; yet in all other respects it is structurally similar to MHC class I and II molecules (42).

While G8 was generated by immunizing Balb/c nude mice with B10.BR spleen cells, KN6 was derived from C57BL/6 thymocytes. As KN6 and G8

show similar fine specificity toward stimulator cells from various recombinant inbred strains of mice, it would be interesting to identify amino acid residues on the T10/T22 molecules that are contacting G8 and then to determine if these residues are also important for the recognition by KN6. Regardless, mice transgenic for the G8 and KN6 TCRs show similar, if not identical, positive and negative selection patterns (6).

3.1.3 GI RECOGNITION BY TGI4.4 Another $\gamma\delta$ T cell clone, TgI4.4, was reported to recognize a herpes simplex virus type 1 transmembrane glycoprotein, gI. Gene transfection was used to demonstrate that gI is recognized only when expressed on the cell surface. Wild-type gI expressed in antigen-processing defective RMA-S cells is recognized by TgI4.4, as is the soluble recombinant gI molecule gI-Ig when coated onto plates (32). Thus, gI is recognized directly without any requirement for antigen processing or presentation by other molecules.

3.2 *Antigen-Processing Requirements in Other Experimental Systems*

In addition to the experimental systems described above, other $\gamma\delta$ T cell specificities have been demonstrated by gene transfection. These are the human $\gamma\delta$ T cell clones specific for CD1 (43) and the classical MHC class I molecules HLA-A2 and HLA-A24 (44, 45). In these cases, genes transfected in mouse and fibroblast lines were all recognized, suggesting that no specific peptide is required for the recognition. Therefore, the situations may be similar to the ones described above. Nevertheless, the precise recognition requirements remain to be determined.

Two well-documented cases indicate that the $\gamma\delta$ T cell recognition may involve "complexed antigen" on the cell surface. Vidovic et al (46) immunized DBA/2 mice with the synthetic copolymer Glu-Tyr (GT) and reported the generation of a $\gamma\delta$ T cell hybridoma that responded to GT only in the presence of stimulator cells expressing the Qa-1^b (but not the Qa-1^a) molecule. The response could be blocked by an anti Qa-1^b antibody. Holoshitz et al (47) identified a human $\gamma\delta$ T cell clone from synovial fluid of a patient with early rheumatoid arthritis. This clone proliferated in response to mycobacterial extracts as well as to fragment C (450 amino acids of the carboxy-terminal end) of tetanus toxin (TT). A synthetic peptide (23 amino acids) corresponding to a subregion of the fragment C can also stimulate this clone. The TT response requires the presence of cells expressing an MHC class II molecule, DRw53, and can be inhibited by an anti-DRw53 antibody. Curiously, it requires a higher molar concentration of the peptide than of the fragment C to induce a similar level of T cell response. This result is in contrast to those $\alpha\beta$ T cells that

recognize processed protein antigens. T cell stimulation is in general much more efficient with peptide. Nevertheless, in these two cases, it remains to be investigated whether the Glu-Tyr polymer and the tetanus toxin are "processed" and if so, what kind of antigen processing is required.

Vγ1-bearing murine $\gamma\delta$ hybridomas secrete lymphokines in response to synthetic peptides representing a short hydrophobic segment of the mycobacterial 60-kDa heat shock protein (48). In these assays, the peptides are added directly to the responder cells in the absence of exogenous presenting cells. Although these responses are TCR dependent, it is not clear if these peptides or some other cell surface structure(s) induced or altered by these peptides are the true ligand for these $\gamma\delta$ T cells (48). Indeed, this peptide induces K^b and D^b as well as Qa-1 expression on RMA-S cells. In addition, because these peptides are poorly water soluble, they have to be added as a slurry, which could make them effectively polyvalent and able to stimulate the T cells by direct TCR cross-linking (48).

Loh et al (49) showed that Jurkat cells transfected with human Vγ9 gene in combination with either Vδ1 or Vδ2 could respond to staphylococcal enterotoxin A (SEA) at a concentration of 1–10 mg/ml. However, this concentration is a 100–1000-fold higher than needed for $\alpha\beta$ TCR stimulation in a similar experimental system (50). If SEA is indeed recognized in this system, it would be interesting to investigate how the recognition occurs and whether the Vγ or Vδ CDR3 regions are involved.

In another system, Kim et al (51) showed that a Burkitt's lymphoma–specific human $\gamma\delta$ CT recognized cells transfected with tumor-specific immunoglobulin light chain genes with and without the leader peptide, and that this reactivity was blocked by an anti-serum against a mitochondrial heat shock protein (grp75). The authors suggested that an immunoglobulin (Ig) light chain–derived peptide was recognized in the context of the heat shock protein. It would be interesting in this system to identify the specific peptide recognized and to test whether antigen-processing defective cells transfected with the light chain gene are still as active as stimulators. Also, as anti-sera are notoriously cross-reactive, it would be important to reproduce the blocking data with a monoclonal antibody to the grp75 protein.

3.3 *$\gamma\delta$ T Cells Can Be Stimulated by Nonpeptide Antigens*

$\gamma\delta$ T cells from healthy human peripheral blood and from patients with tuberculoid leprosy or rheumatoid arthritis respond to heat-killed mycobacteria. Only a minority of these cells respond to mycobacterial heat shock protein. The major stimulatory components in the former have recently been identified as phosphate-containing, nonpeptide molecules. While the consensus is that phosphate is a necessary component, compounds identified from various

Table 2 Nonpeptide mycobacterial antigens that stimulate human Vγ9Vδ2 (Vγ2Vδ2) T cells[a]

Name	Structure	References
Phosphocarbohydrates	Unusual carbohydrates with terminal phosphorylation	Schoel et al (1994) *Eur. J. Immunol.* 24, 1886
TUBag3	X-uridine 5′-triphosphate	JJ Fournié, personal communication
TUBag4	X-thymidine 5′-triphosphate	Constant et al (1994) *Science* 264, 267
TUBag1, 2	X, includes phosphate	
Isopentenyl pyrophosphate	$CH_2 = C(CH_3)CH_2CH_2\text{-}PP_i$	Tanaka et al (1995) *Nature* 375, 155

[a]All compounds have been isolated from myobacterial extracts and require some type of phosphorylation for stimulatory activity. The unknown structure "X", when phosphorylated, is the minimal active stimulatory component of the TUBag compounds. "X" is not a prenyl or alkenyl derivative (JJ Fournié, personal communication).

laboratories with different mycobacteria-responsive clones appear to be distinct in their structures (Table 2).

Schoel et al (34) identified the active component in mycobacterium as a non-protein low molecular weight (1–3-kDa) compound that contains unusual carbohydrate and phosphate moieties. Constant et al (35) identified four distinct but related stimulating agents from *M. tuberculosis* (strain H37Rv), water-soluble extracts termed TUBag 1-4. TUBag 4 was identified as a 5′-triphosphorylated thymidine substituted at its γ-phosphate by an as-yet-to-be-characterized low molecular weight structure. TUBag 3 has a similar structure but contains uridine instead of deoxythymidine. TUBag 1 and 2 are naturally occurring, non-nucleotide, minimal active fragments of TUBag 3 and 4. TUBag 4 can induce expansion of Vγ9Vδ2 T cells from human peripheral blood and stimulate specific $\gamma\delta$ T cells. TUBag 1-4 are found in all mycobacteria species, but in various amounts and localizations. In tuberculosis-related species, all TUBag molecules are associated with the bacterial body, but in nonpathogenic species they are mostly secreted (P Constant, Y Poquet, M-A Peyrat, F Davodeau, M Bonneville, J-J Fournié, in press). Others (36, 37, 52), working with extracts from the same mycobacterial strain (H37Rv), identified isopentenyl pyrophosphate and related prenyl pyrophosphate derivatives as the major stimulatory component. They also found that synthetic alkenyl and prenyl derivatives of phosphate and pyrophosphate as well as γ-monoethyl derivatives of nucleoside and deoxynucleoside triphosphate stimulate particular $\gamma\delta$ T cell clones, with the pyrophosphate and the TTP, UTP γ-derivatives being the most potent. Although the relative biological importance of these compounds remains to be determined, it is clear that a major class of stimulants are phosphate-containing nonpeptides. It is also clear that multiple phosphate-containing compounds are able to stimulate different clones with different efficacy (Table 3).

[1]Nomenclature according to (34, 35). Vγ9 is called Vγ2 in (36, 37).

Table 3 Nonpeptide synthetic antigens that stimulate human Vγ9Vδ2(Vγ2Vδ2) T cells[a]

Anchor chain	Structure	Derivative (μM)[a] Phosphate	Pyrophosphate	UTP
Alkenyl-				
Allyl-	$CH_2 = CHCH_2-$	800	5	4
Crotyl-	$CH_3CH = CHCH_2-$	350	8	3
Prenyl-				
Dimethylallyl-	$CH_3C(CH_3) = CHCH_2-$	30	10	0.3
isopenteyl-	$CH_2 = C(CH_3)CH_2CH_2-$	700	3	4

[a]The values given are the antigen concentration (μM) required for the half maximal proliferative response of the $\gamma\delta$ T cell clone 12G12. Reproduced by permission from Tanaka et al (37).

One important finding is that all of these compounds can be found in both microbial and mammalian cells. Constant et al (35) proposed that the mammalian TTP-X and UTP-X conjugate may be involved in a salvage pathway in DNA and RNA synthesis, and thus they could be involved in a metabolic pathway related to DNA or RNA synthesis, such as cell proliferation. Such molecules would fit with the stress antigen or conserved primitive stimulus expected for $\gamma\delta$ T cell ligands (6).

Tanaka et al (37) proposed that a link in the recognition of both microbial pathogens and hematopoietic tumor cells by these $\gamma\delta$ T cells is provided by the common set of prenyl pyrophosphate intermediates, isopentenyl and related prenyl phyrophosphate derivatives. These compounds are present in normal mammalian cells as precursors in lipid metabolism for the synthesis of farnesyl pyrophosphate. In mammalian cells, farnesyl addition has been proposed to be a critical modification for the membrane association of the ras protein and is required for transforming activity. The observation that some $\gamma\delta$ T cell populations accumulate in lesions caused by mycobacterial infections in humans (53) and are able to respond to virally and bacterially infected cells (52, 53) would suggest that these cells respond to a class of antigens shared by a number of pathogens and transformed, damaged, or stressed cells.

4. STRUCTURAL DIFFERENCES BETWEEN $\gamma\delta$ AND $\alpha\beta$ TCR

4.1 *$\gamma\delta$ TCR Are More Similar To Immunoglobulin Than To $\alpha\beta$ TCR in CDR3 Length Distributions*

Based on conserved amino acid residues and secondary structure predictions, $\alpha\beta$ and $\gamma\delta$ TCRs most likely have an Ig-like structure (54). [X-ray

crystallographic analysis has recently confirmed the Ig-like structure of the TCR β chain V and C domain (55).] Also in common among all of these antigen-specific immune receptors is that they confer specificity against a wide variety of potential pathogens by recombining separate V, (D), J elements into a single Ig or TCR variable domain-encoding exon.

X-ray structural analysis of antibody-antigen complexes shows that the loops created by the V(D)J junction (CDR3) of Ig heavy and light chains are always involved in antigen contact (56–60). Similarly, the CDR3 loop of both α and β TCR chains seems critical for peptide recognition (61). It is therefore reasonable to assume that the analogous regions in $\gamma\delta$ chains also play an important role in antigen recognition. In order to develop a systematic way to compare these regions between immune receptors, Rock et al (62) analyzed the CDR3 length distribution of all known antigen receptor polypeptides from mouse and humans. The results are illustrated in Figure 4.

In Ig light chains, CDR3 regions are short and of conserved length, but those of heavy chain are long with a wide range of lengths. This may reflect the fact that Igs recognize a variety of different antigenic surfaces, from small molecules to large pathogens. For $\alpha\beta$ TCR chains, the CDR3 length distributions are significantly constrained and are about equal in length. This probably reflects the requirement for α and β chains of the TCR to contact both the MHC and bound peptide. Surprisingly, in $\gamma\delta$ TCR, the γ chain CDR3 loops are short with a narrow length distribution, and the δ chain CDR3s are long with a broad length distribution. Therefore, with respect to CDR3 lengths, the $\gamma\delta$ TCR is more similar to Ig than to $\alpha\beta$ TCR. This result is consistent with the experimental results discussed above indicating the Ig-like capacity of a number of $\gamma\delta$ T cells to recognize different antigens directly.

In this context, it has been observed that the frequency of $\gamma\delta$ T cell clones recognizing allogeneic MHC molecules in a mixed lymphocyte reaction is very low (compared to $\alpha\beta$ alloreactive clones), and the majority of these clones show a high degree of cross-reactivity (only rarely seen with $\alpha\beta$ alloreactive clones) [reviewed in Haas (6)]. These observations are consistent with the proposal that $\gamma\delta$ TCR recognition is more Ig-like, focusing on the common features shared by MHC molecules. It is noteworthy that the specificity of LBK5 ($IE^{b,k,s}$ but not IE^{d}) is the same as two previously described anti-IE antibodies (64, 65). In this light, MHC recognition by $\gamma\delta$ T cells may represent a fortuitous cross-reaction with other non-MHC structures that are the physiological ligands for $\gamma\delta$ T cells. This is in contrast to the $\alpha\beta$ T cell's alloreactive recognition of another MHC allele, which is thought to represent cross-reactivity by cells selected to recognize peptides on self-MHC molecules, with the high frequency of such clones reflecting the diversity of the bound peptides.

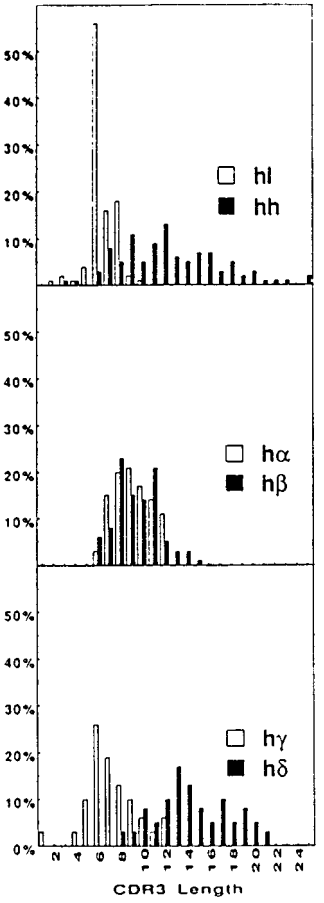

Figure 4 CDR3 length distribution of antigen specific immune receptor chains. An analysis of CDR3 sequences from immunoglobulin $\alpha\beta$ and $\gamma\delta$ T cells by Rock et al (62) illustrates some striking similarities between Ig and $\gamma\delta$ antigen receptors. The Ig heavy chain and the δ chain are long and variable, while the Ig light chain and the γ chain are short and constrained in length. In contrast, the $\alpha\beta$ receptor chains are constrained and similar in length.

Along this line, human $\gamma\delta$ T cell clones from healthy donors that respond to mycobacteria extract are found to express Vγ9 and Vδ2 with variable junctional sequences. This is reminiscent of the Ig receptor usage in naturally occurring mouse B cells that recognize phosphorylcholine. There it was found that only very restricted Ig heavy (VH11, VH12, or Q52) and light chain V gene segments are used, coupled with variable CDR3 junctional sequences (65a). In the latter case, the restricted usage of the V genes is all the more significant since several hundred to a thousand VH gene segments are available to mount an Ig response. The antibody responses to α-1,3 dextran (66) and galactan (67) also show a similar restricted pattern of VH genes, few JH genes, but diverse DH and joining regions in the heavy chain.

By considering all elements that contribute to the variability of the junctional (CDR3) region, such as the numbers of D and J elements used, D element reading frame, junctional diversity, and N region nucleotide addition, Davis & Bjorkman (63) calculated that the number of possible CDR3 sequences was the greatest for $\gamma\delta$ TCR and the least for Ig (irrespective of somatic mutation), and intermediate for $\alpha\beta$ TCR. This suggested that $\gamma\delta$ T cells may have the potential to recognize a wide variety of different antigens directly.

However, the suggestion that $\gamma\delta$ TCR recognition is more Ig-like does not preclude that some $\gamma\delta$ T cells may recognize similar or even the same antigen as do $\alpha\beta$ T cells. One should take note that anti-MHC antibodies can be generated, many of them showing fine specificity toward different subtypes of MHC molecules. Some of these antibodies are even peptide specific (68; P Reay, M Davis, unpublished results).

5. OTHER REQUIREMENTS FOR ACTIVATION THROUGH THE $\gamma\delta$ TCR

5.1 *Multivalence of the Ligands*

$\gamma\delta$ TCR, similar to $\alpha\beta$ TCR, need to associate with CD3 molecules for cell surface expression. Signaling through these receptors requires a multivalent form of the antigen to cross-link the engaged receptors. Cell surface molecules can be recognized as such, but soluble antigen must be rendered polyvalent. A demonstration of this requirement is that in all three cases of $\gamma\delta$ T cells recognizing cell surface molecules—IE^k, T10/T22, and HSV gI protein—a soluble form of the protein can only be recognized when bound to plastic plates. Along this line, the stimulation of mycobacterial extract reactive $\gamma\delta$ T cell clones by small phosphate-containing compounds requires cell-cell contact (Figure 5), even though all cell types tested are able to promote recognition (69, 69a).

The requirement for multivalent antigens would suggest that soluble antigens —such as the phosphate containing compounds—must be associated with certain cell surface molecules for their recognition. It is important to know if the binding and display of soluble antigens is achieved by a variety of different molecules on the surface, or by a limited set of molecules, and if they normally form part of the epitope recognized by the antigen receptors.

5.2 *Signals Through Costimulation and/or Accessory Molecules*

Antigen specific $\alpha\beta$ T cell stimulation requires the engagement of accessory and/or costimulatory molecules on T cells in addition to cross-linking of the engaged antigen receptor. Despite the differences in antigen recognition between $\alpha\beta$ and $\gamma\delta$ T cells described here, $\gamma\delta$ lymphocytes may also depend on two or more signals for full activation.

Among all putative costimulatory molecules present on $\gamma\delta$ T cells, the best studied is the CD28 molecule. Costimulation through CD28 appears to be important for $\gamma\delta$ T cell activation (70). In this context, it is noteworthy that murine skin $\gamma\delta$ cells recognize keratinocytes (71), and that keratinocytes have been found to react with a mAb against BB1 (a member of the B7 family, ligands of CD28) (72). However, as $\gamma\delta$ T cells can be found in a variety of different sites and express different types and different quantities of surface molecules, it is conceivable that they may have different activation requirements with respect to costimulation/accessory molecules.

The CD3 chains, in particular CD3ζ, have been evoked in the signal transduction and activation of T cells (reviewed in 73). However, several lines of

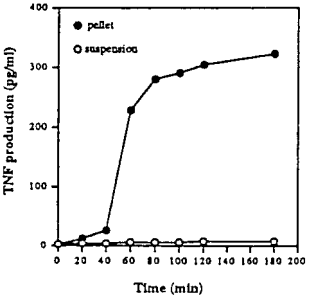

Figure 5 Stimulation of human Vγ9Vδ2 T cells requires cell-cell contact. Stimulation of human $\gamma\delta$ T cell clones by the phosphate-containing TUBag compound from mycobacterial extracts requires cell-cell contact, achieved here by pelleting the cells. T cell response was measured by TNF production. Reproduced by permission from Lang et al (69).

evidence suggest the ζ chain may not be as critical for the activation of some epithelial $\gamma\delta$ T cells. Instead, they may have a propensity for using a Fc receptor chain (reviewed in 6). This implies that the signal transduction requirements and the relative roles of zap70 and syk in this pathway may be different in $\alpha\beta$ compared with some $\gamma\delta$ T cells (A Hayday, personal communication).

6. CONCLUSION

From the experimental results presented here, the differences in $\gamma\delta$ T cell versus $\alpha\beta$ T cell recognition properties provides a basis for the different functions observed for these cells in the immune system. The most striking observations are the lack of antigen-processing requirements for antigen recognition by many $\gamma\delta$ T cells and the likelihood that $\gamma\delta$ and $\alpha\beta$ T cells may recognize different sets of antigens. This suggests that in many cases $\gamma\delta$ T cells can respond to antigens that would not be recognized by $\alpha\beta$ T cells and that they may mediate cellular immune functions without antigen processing. Thus, pathogens, damaged tissues, or even T and B cells can be recognized directly, and cellular immune responses can be initiated without a requirement for antigen degradation or specialized antigen-presenting cells. As indicated by the CDR3 length distributions and the ligand recognition characteristics described earlier, $\gamma\delta$ TCRs as a group may be more "immunoglobulin-like" in their antigen recognition properties. Given the enormous estimated sequence variability of the CDR3 regions of $\gamma\delta$ TCRs, it is likely that these cells may recognize a wide variety of antigens. Such unique properties may allow $\gamma\delta$ T cells to complement effectively the more classical type of $\alpha\beta$ T cell–mediated cellular immunity.

Although the recognition requirements for $\gamma\delta$ T cells are largely derived from observations with model systems, the identification of the mycobacterium antigen stems from physiologically relevant events. We certainly need to test the generality of these rules and to assess the kind of antigens that $\gamma\delta$ T cells are most likely to recognize in other pathological situations. It is important to establish whether $\gamma\delta$ T cells can contribute to pathogenesis and/or protective immune responses, and whether the responses are influenced by the antigen. An approach to this question is to determine in an infectious disease model whether $\gamma\delta$ T cell specificities are directed toward pathogens and/or to damaged tissue, and in the latter case, whether the same antigen is also present in normal undamaged tissue as well.

The issue of $\gamma\delta$ T cell specificity is also important for the understanding of $\gamma\delta$ T cell development. Interestingly, the phosphate-containing compounds isolated from mycobacterial extracts can be found both in pathogens and in mammalian cells. Thus, they are a self as well as a non-self component, yet $\gamma\delta$ T cells with this specificity are not eliminated from the normal repertoire. While

some experiments with $\gamma\delta$ TCR transgenic mice suggest that these T cells are both positively and negatively selected in much the same way as $\alpha\beta$ T cells (6), others show an entirely different mode of selection (74). There are also documented cases where $\gamma\delta$ T cells develop extrathymically (6). It is not clear in those situations what and how exogenous antigens are involved.

It was suggested that "the analysis of antigen recognition may be the key to the understanding of the unique funtions of $\gamma\delta$ T cells" (6). Certainly, this is a major part of the puzzle of $\gamma\delta$ T cell biology. While progress in this aspect has been rapid in the past few years, we still need to know more about what is being recognized and how it is recognized in the $\gamma\delta$ T cell response to immune challenges. We also need to know the consequences of such recognition. In any case, it seems that we are a little further along in the understanding of this novel part of the immune system.

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SALMONELLOSIS: Host Immune Responses and Bacterial Virulence Determinants[1]

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ABSTRACT

The lifestyle of bacterial pathogens requires them to establish infection in the face of host immunity. Upon entering a potential host, a variety of interactions are initiated, the outcome of which depends upon a myriad of attributes of each of the participants. In this review we discuss the interactions that occur between pathogenic Salmonella species and the host immune systems, but when appropriate to broaden perspective, we have provided a general overview of the interactions between bacterial pathogens and animal hosts. Pathogenic Salmonella species possess an array of invasion genes that produce proteins secreted by a specialized type III secretion apparatus. These proteins are used by the bacteria to penetrate the intestinal mucosa by invading and destroying specialized epithelial M cells of the Peyer's patches. This manuever deposits the bacteria directly within the confines of the reticuloendothelial system. The host responds to these actions with nonspecific phagocytic cells and an imflammatory response as well as by

[1]Abbreviations: IFN-γ, γ interferon; TNF-α, tumor necrosis factor α; FAE, follicle-associated epithelium

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activating specific cellular and humoral immune responses. Salmonella responds to this show of force directly. It appears that the bacteria invade and establish a niche within the very cells that have been sent to destroy them. Efforts are underway to characterize the factors that allow these intracellular bacteria to customize intracellular vacuoles for their own purposes. It is the constant play between these interactions that determines the outcome of the host infection, and clearly they will also shape the evolution of new survival strategies for both the bacterium and the host.

1. INTRODUCTION

From moments after birth, we constantly breathe, eat, and come into physical contact with millions of microorganisms until we are consumed by them at death. Some are harmless transients—"just passing through, so to speak." Others become part of the commensal flora that we harbor a lifetime. Many of the billions of bacteria, fungi, and protozoa that make up our normal flora have never been cultivated; numerically we carry more microbial cells than we do cells of our own. We would not survive long without our constant "normal" microbial flora.

Yet we are in a delicate balance with these microorganisms. Indeed, our immune system is in large part a reflection of our need to survive the onslaught of a myriad of microbes that surround us. Let the innate immunity be compromised, and the harmless microorganisms can quickly become serious or even fatal threats to our existence. In addition, among the microorganisms that we encounter each day are those whose survival depends on their ability to cause some degree of cellular damage to their host. It is this group of microorganisms, the pathogens, that are the focus of this review. Because we cannot hope to cover all aspects of microbial interaction with the immune system, we have chosen to examine a single infectious disease, typhoid fever, as an illustrative example. Our focus is on the determinants of bacterial pathogenicity that permit the microorganism to prevail in the face of a normal immune system and on the strategies microorganisms employ to thwart immune surveillance.

A bacterial pathogen must enter a host, find a unique niche, circumvent competing microbes and host defense barriers, and multiply sufficiently to establish itself or to be transmitted to a new susceptible host. Pathogens damage their host by intoxication or compromise of the integrity of cells. In most cases the damage is not serious (subclinical disease in humans), but a proportion of hosts can be expected to suffer from overt disease or even be killed. The focus of our attention, *Salmonella typhi*, is an enteric bacillus that can trace its

ancestry to an ancestor common with *Escherichia coli* about 120–160 million years ago, around the time of the origin of mammals. The first Salmonellae parasitized reptiles and birds as well as mammals. Many salmonella strains exhibit a remarkable degree of host-species specificity. *S. typhi* infects humans and other higher primates, exclusively, to cause a systemic infection. *S. typhimurium* causes a similar systemic ailment in mice but can also infect a variety of other animals to cause nonspecific, usually short-lived, superficial gastroenteritis.

Upon entering a suitable host, Salmonella species like *S. typhi* that are host-adapted establish infection by sequentially surviving the acidic pH of the stomach, competing with the normal bacterial flora of the small bowel, and passing through the epithelial barrier to proliferate in the Peyer's patch and the draining mesenteric lymph nodes. In an immunologically naive host, the primary barriers to successful bacterial replication are the nonspecific antimicrobial factors like complement and phagocytic cells, both polymorphs and macrophages. If this initial encounter with the hostile environment of the host is successful, these microorganisms subsequently drain through the lymphatics to the thoracic duct into the blood and ultimately infect the liver and spleen. To cope with these different environmental conditions, the bacteria possess different sets of genes that are alternately switched on and off, as needed, by regulatory systems that sense the surroundings of the bacteria. This coordinated gene regulation ensures expression of bacterial virulence factors at the proper stage of infection, while preventing the unnecessary expenditure of energy that might compromise the ability of Salmonella to establish infection of the host. It can be seen in each step of the pathogenesis of infection that the microbe takes its cues from its encounters with both innate and specific elements of the immune system. How the microbe succeeds tells us a good deal about both the elements of bacterial pathogenicity and the function of the immune system.

The outcome of infection depends upon a number of factors including the size of bacterial inoculum, the virulence of the infecting Salmonella strain, medical intervention, and the ability to mobilize immune defenses to respond to the infection. Numerous studies have demonstrated that humans as well as experimental animal hosts respond to Salmonella infection by activating both humoral and cell-mediated immune responses. However, since activation of primary immunity often takes longer than bacterial growth affords, the development of protective immunity through vaccination has been of great significance in areas of the world where typhoid fever is endemic. While we may know how to immunize to prevent infection, we do not necessarily understand very much about either the pathogenesis of the microbial disease nor the capacity of our immune system to prevent disease.

2. THE HOST-PARASITE INTERACTION

2.1 *Typhoid Fever*

Human typhoid fever (enteric fever) is a severe systemic infection of the reticuloendothelial system caused by the bacterium *Salmonella typhi*. Survey data from the WHO Diarrheal Disease Control Program in 1980 estimated that more than 12 million cases of typhoid fever occur each year (1), most in areas of the world where sewage disposal is poor and the water supply is not properly purified. Prior to the availability of antibiotics, mortality from typhoid fever was high worldwide, with approximately one in seven (15%) patients dying from infection (1, 2). In the modern era, the administration of early antibiotic treatment has proven to be highly effective in eliminating disease; however, significant numbers of typhoid patients continue to die in underdeveloped parts of the world because of the unavailability of appropriate medical care. In certain areas of Africa and Asia, typhoid mortality rates as high as 30% have been reported, apparently because antimicrobial therapy is greatly delayed, if administered at all (3, 4). In contrast, < 1000 cases of typhoid fever are reported in the United States each year, and less than 1% of these cases result in death (5)—statistics that highlight the importance of access to medical care as well as proper hygiene.

The onset of disease takes from 5 to 20 days depending on the health and immune status of the individual and the number of organisms ingested. Often the first signs of infection are enterocolitis with diarrhea, frequently accompanied by the nonspecific symptoms of headache, chills, anorexia, weakness, dizziness, muscle pains, and/or abdominal cramping. Typically, these symptoms resolve before the classic indicators of typhoid fever appear: abdominal pain and fever. The symptoms of untreated typhoid fever begin to resolve by the fourth week of infection, although relapse occurs in $\approx 10\%$ of individuals apparently recovering from the infection (6). Intestinal complications can occur in the later stages of untreated infection; these include bleeding and perforation that are the result of an inflammatory response in the Peyer's patch, followed by necrosis and ulceration of the intestinal epithelium (7–10). Intestinal perforation usually results in peritonitis and often requires surgical intervention. In contrast, appropriate antibiotic therapy normally resolves *S. typhi* infections within 3–5 days and prevents the occurrence of most complications.

3. THE IMMUNE RESPONSE TO SYSTEMIC SALMONELLA INFECTIONS

The majority of individuals that survive typhoid fever generally acquire immunity to future infection, although the incidence of recurrence can be as high

as 2–3% if an individual is subsequently reexposed to a high inoculum of organisms or received early antibiotic therapy for the initial infection (11). A number of studies have been performed to identify the components of the human immune response to typhoid fever. In adult volunteers who received orally administered *S. typhi* vaccine strain Ty21a, measurement of antibody responses indicated that significant levels of IgA and IgG were induced against *S. typhi* lipopolysaccharide (LPS) (12). It was not clear, however, whether these antibody responses contributed to protective immunity. Another study examined the efficacy of purified Vi antigen as a vaccine in a field trial in Nepal (13). Individuals that received the Vi capsular polysaccharide vaccine had a typhoid fever attack rate of 4.1%, while the control group had a 16.2% attack rate. In addition, the group receiving the Vi antigen vaccine had a significant increase of serum antibody to the Vi antigen. Cell-mediated immune responses to live *S. typhi* have been documented as well (14, 15). Seventeen volunteers vaccinated with Ty21a, in an in vitro killing assay (16), had T cell lymphocyte populations in their serum that inhibited the growth of *S. typhi*. The antigen(s) responsible for stimulating this cellular immunity was not identified, but the authors postulated that an antibody-dependent cellular cytotoxicity mechanism might be responsible for the levels of bacterial killing observed.

A significant percentage (1–6%) of typhoid patients become chronic carriers of *S. typhi* (17), as do many people who have never had a clinical history of typhoid fever (18). These individuals shed bacteria in their stools or urine for periods of time greater than 1 year without any signs of disease. Typhoid carriers are of special concern from a public health point of view because they are the reservoirs for the spread of the disease (19). Is the carrier state the ultimate goal of *S. typhi* infection? The organisms are carried for years even in the presence of a brisk immune response; chronic carriers of *S. typhi* have high levels of circulating Vi antibody in their serum (20) as well as O- and H-specific antibodies. The organism has established a privileged niche, sequestered from the host immune defenses, ideal for continued low levels of continous growth. If we understood the chronic carrier state, we would obtain considerable insight into the bacterial strategy for survival as well as how we might overcome not only typhoid but other persistent microbial infections as well.

There is no suitable animal model for typhoid fever. Most investigators have instead focused on the analogous disease produced in mice by *S. typhimurium*. Different strains of mice show different levels of susceptibility to salmonella infection. While resistance to disease, whether in animal or human is multigenic, there has been considerable emphasis on the *Ity* gene (21) (also *Bcg* and *Lsh* loci) (22) that has a major effect on susceptibility to salmonella infection. Mice are either salmonella-sensitive Ity^s, or they carry the dominant Ity^r allele.

Ity^s mice succumb to overwhelming sepsis following the parenteral injection of very few microorganisms. The major effect of *Ity*^{r} is an almost complete inhibition of bacterial growth in mice (23).

In vitro killing studies suggest that the *Ity* effect is due to differences in the rate of intracellular killing of salmonella by resident phagocytic cells (24, 25). The mechanism by which *Ity*^{r} macrophages exert their enhanced bactericidal activity was not clear, although they showed enhanced expression of surface markers (Ia and Acm-1 antigen) associated with the state of activation (26) and in the production of oxgyen and nitrogen radicals in response to secondary stimuli like interferon-γ (IFN-γ) (27). The candidate gene product of *Ity*, designated *Nramp*, is expressed exclusively in macrophage populations from reticuloendothelial organs and in the macrophage line J774; it encodes an integral membrane protein that has structural homology to transport systems, suggesting that *Ity* encodes a macrophage-specific membrane transport function (28).

One advantage of using an *Ity*^{s} mouse strain when studying Salmonella immunity is that both humoral and cell-mediated immunity are required for protection in these mice, while innately resistant mice control low doses of virulent Salmonella with a moderate, nonspecific immune response (29–31). One defect of *Ity*^{s} mice, in comparison to *Ity*^{r} mice, is that the levels of IFN-γ produced in response to challenge by the pathogenic organisms are reduced (32, 33). Ramarathinam et al extended this finding by demonstrating that the natural killer (NK) cell population of mice produce IFN-γ in response to *S. typhimurium* infection and that this response is muted in *Ity*^{s} mice (34). Early work established that IFN-γ and tumor necrosis factor *alpha* (TNF-α) play a role in host responses to Salmonella infection. Nonphagocytic tissue culture cells display an increased resistance to invasion when treated with these cytokines (35), and IFN-γ increases the fusion of phagosomes containing bacteria with lysosomes (36).

The primary role of IFN-γ appears to be the inhibition of bacterial growth rather than induction of host-mediated killing mechanisms (37, 38). Exogenously supplied IFN-γ decreased the numbers of bacteria found in the spleen early in infection but had no effect at later times. Treatment of mice with anti-IFN-γ antibody abolished the ability of mice to clear sublethal doses of organisms. The lymphoid cells within intestinal Peyer's patches produce large amounts of IFN-γ in response to stimulation with *S. typhimurium*, indicating that this cytokine plays an important role in the host response to infection (39). Results of other experiments indicate that the levels of TNF-α also increase in response to infection with *S. typhimurium* (40). Inhibition of the TNF-α response increases the susceptibility of the mouse to infection. Both IFN-γ and

TNF-α appear to play important roles in stimulating early host defenses against virulent *S. typhimurium*. Transgenic mice that no longer produce IFN-γ (GKO mice) and TNF-α should greatly facilitate efforts to dissect the contributions of these cytokines to host immunity to Salmonella infections as well as to other enteropathogens (41, 42).

The host immune response to virulent Salmonella appears to induce a form of immunosuppression. Mice vaccinated with an attenuated strain of *S. typhimurium* become immune to future challenge with virulent *S. typhimurium* but are unable to mount immune responses, 1 to 3 weeks postimmunization, against non-Salmonella antigens (43). Phagocytic cells mediate this phenomenon because depletion of macrophages from cell suspensions in vitro abolishes this effect. Further investigation revealed that Salmonella-activated macrophages produce large quantities of nitric oxide that poison lymphocytes (44). This nitric oxide effect can be reversed by treating mice with IL-4 or anti-IFN-γ monoclonal antibody. A reduction in numbers of functional lymphocytes appears to be the direct cause of the suppression of the mouse immune response observed in systemic murine typhoid (45).

Other work has measured the primary antibody response in mice to *S. typhimurium*. The humoral response to *S. typhimurium* in mice parallels the antibody response observed in human typhoid fever, which is directed against LPS and a number of other undefined antigens (46, 47). Transfer of immunity experiments have demonstrated that $CD4^+$ cells, $CD8^+$ cells, and serum are all required to protect naive mice from challenge with virulent *S. typhimurium* (31). These results emphasize the importance of both humoral and cellular immunity in controlling pathogenic Salmonella infections.

4. INTERACTIONS OF INVASIVE SALMONELLA WITH THE HOST EPITHELIAL BARRIER

The penetration of the intestinal mucosal barrier is an essential step in Salmonella pathogenesis. Carter & Collins (48) were the first to demonstrate that invasive *S. typhimurium*, introduced into the stomachs of mice, became associated primarily with the lymphoid follicles, or Peyer's patches, of the small intestine rather than with the intestinal wall. Another study confirmed these results by demonstrating that significant numbers of *S. typhimurium* could be isolated from Peyer's patch tissue within 3 h of gastric inoculation of mice (49).

Recent research findings have shed further light on the Salmonella route of entry into the host. The follicle associated epithelium (FAE) of mice is composed of $\approx$5–10% of specialized epithelial M cells (50, 51); the remainder of the cells are enterocytes (52). M cells are unique epithelial cells that form

tight junctions with neighboring enterocytes, have an increased pinocytic activity, and are believed to have the function of priming intestinal immunity by engulfing antigenic samples from the gut and delivering them to the lymphoid cell population that resides within the follicle (53–61). Based on previous findings that the Peyer's patches are the initial site of Salmonella invasion, several laboratories have addressed whether M cells might be the specific targets of invasive Salmonella strains for initiation of infection. One group reported that pathogenic *S. typhi* attached to and destroyed the M cells of ileal Peyer's patches in mice, without any apparent invasion of the organisms into the cells (62). Two recent studies have examined the interactions of the mouse pathogen *Salmonella typhimurium* with murine intestinal tissue using a ligated loop model (63, 64). In each case, invasive *S. typhimurium* specifically invaded the M cells of the lymphoid follicles within 30 min of introducing microorganisms into murine intestinal loops; there was no apparent interaction with adjacent enterocytes during this early phase of infection (Figure 1). Bacterial entry of the M cells occurred by rearrangement of the apical membrane of the cell (ruffling) in a manner identical to that seen for invasion of the guinea pig ileum (65) and for tissue culture cells (66–71). However, in contrast to other cell types, M cell internalization of the invasive organisms had a destructive effect on the cytoskeletal architecture that was permanent.

Jones et al also examined the interactions between the bacteria and the Peyer's patch tissue at later times (63). Disintegrating M cells were often seen detaching from the epithelial surface 60 min after the addition of bacteria to the ligated loops. The invading bacteria do not seem to replicate to any significant extent within the M cells, but they subsequently interact with the underlying cell populations of the follicle dome. Virulent Salmonella are well known for their ability to induce their own uptake into macrophages (discussed below), and these bacteria are also capable of invading B cell and T cell tissue culture lines (72). While adjacent enterocytes are spared at early times after infection, by 2 h post-infection there is significant invasion of enterocytes, and the invading microorganisms can be seen moving unhindered into deeper tissue structures. Enterocyte invasion is a classical feature of Salmonella infection of laboratory animals (65) and, of course, of Salmonella food poisoning (73). It is not clear at this time whether nonhost-adapted Salmonella serovars of the type that cause common food poisoning of humans actually initiate infection by the M cell route.

The essential feature of M cell invasion in the pathogenesis of Salmonella infection is seen from the observation (63) that two independent *S. typhimurium* mutants, selected because they could no longer invade cultured epithelial cells, could not cross the epithelial barrier in the ligated loop model; they were also avirulent when fed to animals. Yet, these same mutants when injected

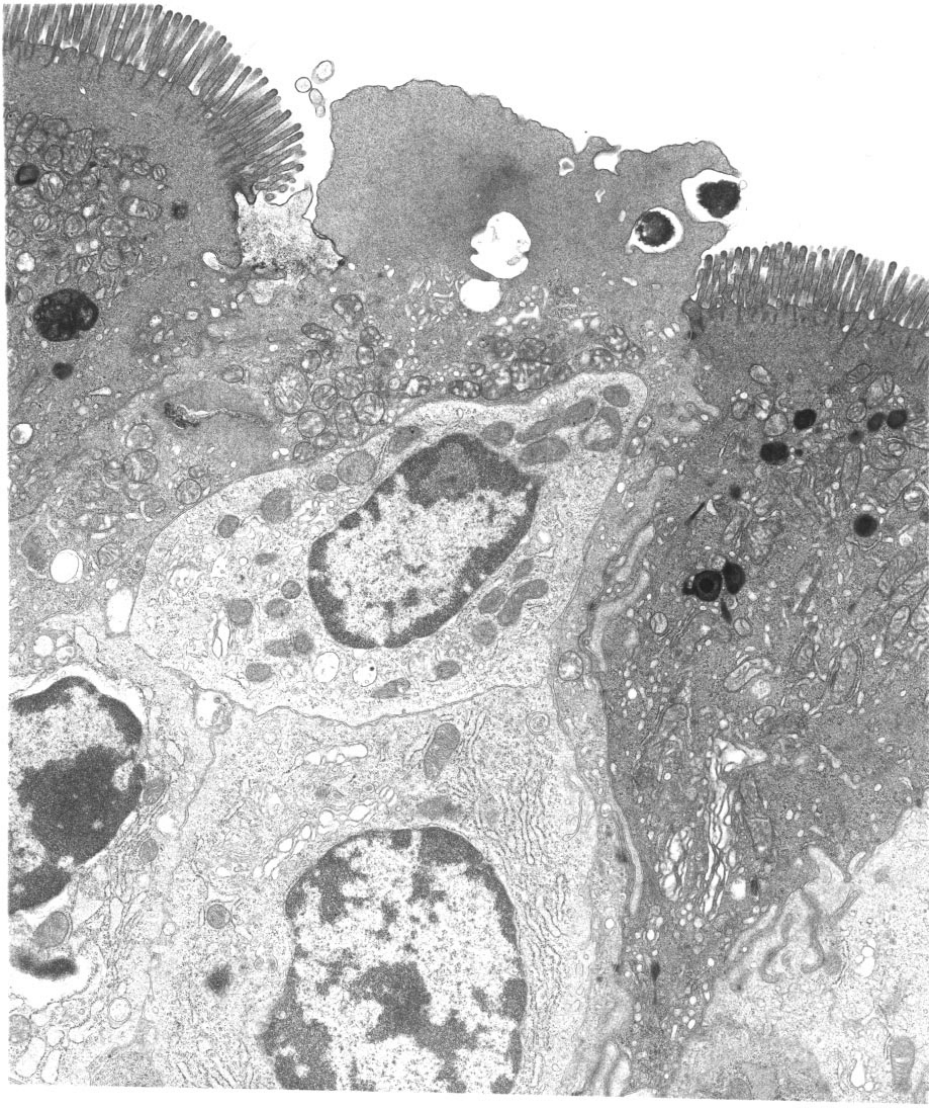

Figure 1 Electronmicrograph of pathogenic *Salmonella typhimurium* organisms invading an ileal M cell 30 min after infection of the murine intestinal tissue. Lymphoid cells are seen immediately beneath the M cell, which has visible rearrangements of its apical membrane.

intraperitoneally were perfectly virulent. Other published work indicates that *S. typhi*, but not the bird-adapted strain *S. gallinarum*, can also enter murine M cells with a characteristic ruffling of the cell surface (73a). However, the frequency of M cell penetration is considerably lower, and the M cell is not killed in the process. Within 2 h of *S. typhi* infection, few bacteria survive, although disintegrating bacilli can be seen in mononuclear cells in the follicle dome.

It is noteworthy that the first specific event of Salmonella pathogenesis drives directly to the heart of host mucosal immunity. This suggests that from the outset, the microbial strategy for survival entails modifying the early determinants of host cell immunity. Host-adapted Salmonella have a relatively prolonged incubation period that gives rise to a muted mononuclear response, as contrasted to the fierce inflammatory nature of nonhost adapted salmonella gastroenteritis. *S. typhi* infection is often followed by a carrier state and spread from individual to individual; human infection by nontyphoidal Salmonella is usually short-lived, and there are few secondary cases. The entry of Salmonella into M cells likely betrays a measured bacterial action honed by evolution for interaction with a more limited repertoire of cell types and designed by the bacterium to modulate the host immune response. Salmonella is not unique in seeking the M cell as its portal of entry. Other bacteria, viruses, and protozoa share this bold step to initiate infection.

5. THE CELLULAR RESPONSE TO SALMONELLA INVASION

While M cells have yet to be cultivated in vitro, the interactions between invasive Salmonella and mammalian cells can be duplicated in vitro using tissue culture cells (66–71). The most prominent visible feature of the entry event, in both M cells and cultured epithelial cells, is the induction of a large membrane ruffle on the host cell membrane that moves outward to enclose the bacterium. Video microscopy has been used to demonstrate that Salmonella-induced ruffling can occur within one minute of attachment to the cell, if the bacteria are grown so that they are primed for entry (67). The bacteria apparently activate a general ruffling or phagocytic pathway since noninvasive bacteria and even latex beads are taken up following stimulation of ruffling by invasive *S. typhimurium* (68). Shortly after entry, an internalized bacterium can be found within a cell with a cytoarchitecture that appears normal.

Tissue culture invasion assays have been used to identify various components of the host cell response to Salmonella entry. Salmonella species are unable to enter mammalian cells that have been previously treated with cytochalasins (74–76). Cytochalasins specifically inhibit microfilament formation by

blocking the polymerization of actin monomers (77, 78). In addition to actin polymerization, Salmonella entry is accompanied by the accumulation of the cytoskeletal proteins α-actinin, talin, tubulin, tropomyosin, and ezrin (79). Drugs such as ammonium chloride and methylamine that prevent acidification of host cell endosomes have no effect on Salmonella entry (74), although there does seem to be an effect on the ability of the organisms subsequently to survive within macrophages (M Rathman, S Falkow, unpublished observation). Others have reported that calcium fluxes occur simultaneously with the Salmonella entry process (69, 80, 81).

There is considerable interest in identifying the signaling pathway used by invasive Salmonella to enter mammalian cells. The effect of genistein, a tyrosine protein kinase inhibitor (82), on the ability of invasive bacteria to enter cells has been examined (83, 84). Genistein effectively blocked entry mediated by Yersinia invasin and uptake of enteropathogenic *E. coli* but had no effect on the entry of invasive *S. typhimurium* into HeLa, Henle-407, and A413 tissue culture cells. Other work has studied the role of the small GTPases, rac and rho, which regulate actin rearrangements such as stress fiber formation and membrane ruffling induced by growth factors (85, 86). Blocking the functions of rac and rho in vivo had no effect on the ability of invasive *S. typhimurium* to induce membrane ruffles and enter a variety of tissue culture cells (71). These data indicate that invasive *S. typhimurium* initiates entry by a rac- and rho-independent pathway. However, it is clear that many distinct pathways independently activate the central MAP kinase regulator as part of signaling cascades (87, 88). Salmonella internalization signal may likely activate an undescribed pathway that acts through MAP kinase to induce membrane ruffling and bacterial internalization (89). Answers to questions about the Salmonella signaling pathway that lead to entry await future experiments.

In broad context Salmonella have learned the nuances by which eukaryotic cells transduce extracellular cues into intracellular responses. Many steps of this cascade are conserved, and homologies have been discovered from yeast to human. Salmonella is not unique in its ability to "understand" the cell biology of its host in such fine detail. Shigella also induce a marked rearrangement in the host cell cytoskeleton that is likewise manifested as cellular ruffling leading to their internalization. Similarly, almost all known enteric pathogens have learned how to trigger a host signal pathway(s). Just as each pathogen occupies a rather distinct niche within the host, so does each have a distinctive way to circumvent the host defense mechanisms in which their attachment to the host cell surface is marked by the phosphorylation of one or more host cell proteins with a coincident modulation of the host cell response in favor of the offending microorganism. A further shared strategy (see below) is the contact-dependent

assembly of proteins on the bacterial surface that acts in concert to bring about interaction with the host cell. If we view our immune system as the evolutionary by-product of survival against an onslaught of microbes, it is perhaps not so surprising to learn that the bacterial strategy for survival is the evolutionary by-product of learning to survive against increasingly sophisticated immune defense mechanisms.

6. SALMONELLA SURVIVAL WITHIN THE LYMPHATIC SYSTEM

Following passage through the epithelium of the Peyer's patch, virulent Salmonella strains enter the environment of the follicle dome, which is populated with host lymphocytes and macrophages. To move into deeper tissue, these bacteria must be able to avoid and/or survive the oxygen-dependent and oxygen-independent killing mechanisms of professional phagocytes following internalization. Some oxygen-dependent mechanisms involve the production of superoxide anions, hydrogen peroxide, hypochlorite, and hydroxyl radicals that are pumped into the phagosomes. *S. typhimurium* strains carrying mutations in recA and recBC are more sensitive to macrophage killing in vitro and are attenuated for virulence in mice (90). Experiments suggest that these virulence defects were caused by the inability of the mutants to respond to superoxide. Presumably, superoxide induces bacterial damage that is repaired by the recA and recBC systems.

Oxygen-independent killing mechanisms include acidification of the phagolysosome and the introduction of degradative enzymes and small bacteriocidal peptides known as defensins. Some microbial pathogens prevent fusion of phagosomes and lysosomes to avoid exposure to the toxic contents of the lysosome, while others have evolved strategies to protect themselves from such an environment (91). In an effort to determine the intracellular survival strategy used by Salmonella, Buchmeier & Heffron (92) compared the frequency of phagosome-lysosome fusion in macrophages infected with either *E. coli* or *S. typhimurium*. Their results indicate that *S. typhimurium* significantly inhibits phagolysosomal fusion and that dividing organisms are primarily found within unfused vesicles. Another group observed that acidification of phagosomes containing live *S. typhimurium* was delayed 4 to 5 hours, while acidification of vacuoles with dead organisms occurred within 1 hour (93). Garcia et al have published findings characterizing the epithelial cell vacuole containing intracellular Salmonella (94).

Fluorescent staining experiments demonstrated that upon entering an epithelial cell, membrane-enclosed bacteria fused with lysosomal membrane

glycoprotein (lgp)-containing compartments but not vesicles containing cathepsin D or the mannose-6-phosphate receptor. The frequency of fusion of Salmonella-containing vacuoles with vesicles containing fluid-phase markers was greatly reduced. Similar observations for the trafficking of *S. typhimurium* in cultured and bone marrow-derived macrophages have been made (M Rathman, SJ Nelson, S Falkow, unpublished data). The invading bacteria almost immediately reside in vacuoles rich in lgp and Rab 7 and Rab 9, members of the Ras-related group of small GTP-binding proteins. These proteins partially confer specificity to membrane targeting and recognition, and they contribute to the control of vesicle fusion events. Salmonella that survive the initial meeting with the macrophage do not encounter cathepsin or mannose-6-phosphate, and they go on to replicate to a limited extent within cells. The capacity of the bacteria to reside within this privileged intracellular niche is dependent upon the prompt acidification of the vacuole. If this is prevented, the bacteria cannot increase in number, although they will survive for at least 24 h. Similarly, Abshire & Neidhardt (95) published work aimed at determining the growth rate of *S. typhimurium* within host macrophages. Their results indicate that the intracellular population of bacteria changes with time. Immediately following invasion, the bacterial population is sensitive to treatment with chloramphenicol and ampicillin, which dramatically reduce the numbers of bacteria that could be recovered on agar plates. However, 2 h after invasion, chloramphenicol and ampicillin were no longer bacteriocidal but were bacteriostatic, indicating that the intracellular bacteria no longer needed protein synthesis to survive and that they were no longer growing rapidly.

The work by Abshire as well as unpublished video microscopy studies by the Falkow lab (D Monack, B Rampach, S Falkow, unpublished observations) indicate that populations of salmonella that enter into cells are not uniform. Many of the bacteria can be seen lysed within vacuoles 30 min after entry. Others go on to inhabit "spacious vacuoles" (96). It is this population that may replicate to some limited extent and perhaps be metabolically transformed for survival within the hostile reticuloendothelial system. These results further suggest that the intracellular environment of the macrophage requires adaptation on the part of the bacteria. Those that express the proper genes survive; those that do not are eliminated by host defenses. Buchmeier & Heffron (97) have examined the effect of the intracellular environment of macrophages on *S. typhimurium* gene expression. They found that the synthesis of over 30 bacterial proteins was induced by exposure of the bacteria to cultured macrophages, including the heat shock proteins GroEL and DnaK. It seems probable that many of these gene products are employed in protecting the bacteria against the diverse killing mechanisms of macrophages and in circumventing host defense mechanisms.

7. THE GENETIC AND MOLECULAR BASIS OF EPITHELIAL CELL INVASION

Clearly, an important component of Salmonella pathogenesis is the ability of the bacteria to transport themselves through the membranes of host cells, including M cells, enterocytes, lymphocytes, and professional killing cells. Identifying the genes required for this process is a focal point of contemporary microbial pathogenesis research. The ability to identify and study Salmonella factors required for entry through the cell membrane has been greatly facilitated by the development of an in vitro tissue culture cell invasion assay. Giannella et al (98) established that the ability of *S. typhimurium* strains to invade mammalian cells in vitro and their ability to pass through rabbit intestinal epithelium were directly correlated. Several research groups, using tissue culture invasion assays, have now begun to identify genetic elements that play a role in the Salmonella entry mechanism. Galán & Curtiss identified a group of four genes, designated *invA, -B, -C, -D,* that conferred the ability to enter tissue culture cells to a noninvasive laboratory strain of *S. typhimurium* (99). Invasive *S. typhimurium* strains carrying *invA* mutation were found to be noninvasive in a tissue culture invasion model and significantly reduced in their ability to cause disease in mice following oral inoculation. The same *invA* mutant strains were fully virulent if mice were inoculated using an intraperitoneal route of infection. These results suggest that the invasion machinery of Salmonella species is important for establishing infection by the oral route. DNA sequence analysis of chromosomal regions adjacent to the *S. typhimurium invA, -B, -C, -D* genes has led to the identification of several more *inv* genes that play a role in Salmonella entry (69, 100, 101). In fact, it appears that a 40-kb segment of DNA carrying the *S. typhimurium* invasion genes has inserted between the *srl* and *cysC* genes at 59 min on the chromosome. This DNA is absent from the corresponding region in *E. coli* (102).

Many of the Salmonella invasion proteins are related at the amino acid level to the type III secretion family of proteins (69, 101, 103, 104) from bacterial species such as *Pseudomonas solanacearum* and *syringae, Rhizobium fredii, Erwinia amylovora,* as well as Yersinia and Shigella species systems (105). As many as 20 proteins are required in these secretion systems for processes such as nodulation specificity, flagellar biosynthesis, and the secretion of virulence factors. The plant-pathogenic bacteria use the type III secretion system to export proteins that cause disease on susceptible plants and to induce a hypersensitive response on resistant plants (105). In Shigella, it is clear that the *spa/mxi* genes that comprise this secretion system are required for the export of the Ipa proteins that mediate entry into the host cell. The type III secretion system in Yersinia exports the cytotoxin YopE and seems to be triggered

when the bacterium comes into contact with a host cell (106). In Salmonella, the invasion secretion apparatus appears to be required for the assembly of large appendages on the surface of the invasive bacteria that may play a specific role in inducing the membrane ruffling that leads to bacterial uptake (107). The InvJ (SpaN) protein is exported by the *S. typhimurium* secretion system (108). This protein has limited homology with the EaeB protein of enteropathogenic *E. coli* that is involved in actin condensation. These observations are both exciting and intriguing, and they provide the basis for a more thorough study of the invasion mechanism. A diverse group of bacteria has evolved to use a common secretory apparatus (type III) for highly specialized purposes such as flagellar biosynthesis and interactions with plant and animal cells. Interestingly, high antibody titers to the Shigella Ipa proteins have been found in the serum of patients recovering from shigellosis (109). Since homologous systems function in both Shigella and Salmonella, it seems likely that the Salmonella proteins that stimulate bacterial internalization might also stimulate antibody and/or cellular immune responses.

Other Salmonella invasion loci have been identified based on regulation of lac fusions. As Salmonella strains enter a host, the organisms must sense and respond to changing conditions if they are to take advantage of their new environment. Accordingly, the expression of the Salmonella invasive phenotype is regulated by physiological conditions, such as osmolarity, growth state, and oxygen availability, similar to those found in the lumen of the bowel (110–113). Some of the invasion genes that respond to these environmental cues have been identified and characterized (114–116). Recent characterization of one of these genes, *hilA* (117), has determined that this gene encodes a positive transcriptional regulator related to OmpR and ToxR (118, 119). The HilA protein activates the expression of invasion genes found at 58 min on the *S. typhimurium* chromosome. Two of these genes, *prgH* and *orgA*, map adjacent to the *hil* gene on the Salmonella chromosome (114, 115). The regulation of the *prgH* gene appears to be complex as it is also repressed by the *phoP/phoQ* two component regulatory system that regulates *S. typhimurium* genes necessary for intracellular survival in macrophages (120–122). An isogenic *prgH S. typhimurium* mutant is $\approx$10-fold less invasive than is the wild type in tissue culture invasion assays, and the mutant is less virulent for mice following oral or intraperitoneal inoculation. The loss of virulence by either route of inoculation suggests that the *prgH* gene product plays a role in both mucosal invasion and survival of the pathogens as they interact with phagocytic cells. Another group of genes has been identified by screening for oxygen-regulated *lacZY* transcriptional fusions that have lost the ability to enter tissue culture cells (115). One of these genes, *orgA*, is regulated by the *hilA* positive

regulator. Sequence analysis of the *orgA* open reading frame failed to provide any information about the possible role of the OrgA protein in invasion. However, the virulence of an *orgA S. typhimurium* mutant has been tested in a mouse model of typhoid fever. This mutant had the same virulence phenotype for mice as *S. typhimurium invA* mutants; the strain is $>$ 60-fold less virulent for mice following oral inoculation but is as virulent as the wild-type strain using an intraperitoneal route of infection.

One of the most remarkable conclusions that has emerged from the search for the genes controlling microbial "invasion" is the high degree of shared genetic homology among virulence genes seen among apparently different pathogens. Salmonella, Shigella, the enteropathogenic *E. coli* (EPEC), and Yersinia all share a remarkably similar type III secretion apparatus that leads to the contact-dependent secretion of one or more functional gene products, and this has a profound effect on the host cell. Each secretion system has its distinct focus, however. For example, while both Salmonella and Shigella invade host enterocytes with a ruffling flourish, Salmonella does so through the apical surface of the cell; Shigella however is restricted to the basolateral aspect of the host epithelial cell. EPEC is content to attach firmly to the surface of host enterocytes and to modify the cytoskeletal architecture so that it forms a tight pedestal of actin, which serves as a nucleation point for the growth of a minute colony of bacteria on the surface of the cell; in the process the surface of the enterocyte is effaced. Yersinia, like Salmonella, firmly binds to the host cell surface through one or more β-integrins. In response to both temperature and low Ca^{++} cues, Yersinia secretes a protein that is inserted into the host cell plasma membrane, through which it further secretes catalytic products, like a tyrosine phosphatase that nullifies the ability of cells to signal a warning. If attachment occurs to a phagocytic cell, the Yersinia strategy destroys the capacity of the cell to carry out phagocytosis. Is this commonality of genetic structure a reflection that all of these bacteria are targeted for an initial encounter in the small bowel of their preferred host animals? Perhaps. The basic repertoire of genes of the original facultative gut commensal that was the ancestor of all of these enteric bacilli permits only a certain limited degree of flexibility for surviving an encounter with the host immune system.

Another striking finding that has emerged from the examination of the genetic basis of bacterial virulence has been the concept of the "pathogenicity island." Simply stated, the genetic and molecular analysis of virulence genes in EPEC, uropathogenic *E. coli*, *Yersinia pestis*, and most recently Salmonella has clearly shown that the virulence genes are clustered on the bacterial chromosome as a single large insertion that is missing from nonpathogenic representative members of the same bacterial species. For example, *E. coli* K-12 completely lacks

either of the two insertions of 70 Kb or 190 Kb that are found alone or together in many uropathogenic Escherichia strains. Similarly, EPEC strains possess a 40-Kb insertion of genes that is missing in K-12. Genome sequencers beware! What is intriguing is that, in several cases, the insertion of these blocs of pathogenicity genes appears to have occurred at the same site.

The full description of the pathogenicity island concept is beyond the scope of this review. However, it is consistent with the idea that pathogenicity has evolved in large genetic quantum jumps rather than by slow adaptive evolution (123). In the Salmonella, both nontyphoidal and typhoid strains possess a similar pathogenicity island located between minutes 58–60 on the chromosome. This bloc of genes, as noted, seems concerned with entry into cells and the initial encounter with the host immune system. This is likely not the only large insertion in Salmonella concerned with the pathogenesis of infection. David Holden and his colleagues have recently identified an additional pathogenicity island near minute 28 on the Salmonella chromosome that is involved with the capacity of the bacteria to reach the spleen following entry (124). It is intriguing that the preliminary analysis of these genes suggests a distinct, but related, type III secretion system. There is no clear-cut genetic insight into the striking host-specificity exhibited by different Salmonella species. Perhaps a clue will emerge from the differences seen between the genetic maps of *S. typhimurium* and *S. typhi* (125).

8. SURVIVAL WITHIN PHAGOCYTIC CELLS

Efforts to analyze the interactions of pathogenic Salmonella with phagocytic cells have relied on mutants that can be studied with in vitro assays and animal models. One study examined the role of the acid tolerance response (ATR) global regulatory system that protects Salmonella from acidic environments below pH 4.0, such as in the phagosome (126, 127). Two of the genes involved in this response are *atp*, an $Mg2^{+}$-dependent proton-translocating ATPase, and *fur*, an $Fe2^{+}$-binding regulatory protein. Strains with mutations in each of these genes were tested for virulence in a mouse model (128). The *S. typhimurium atp* mutant was avirulent when administered either orally or intraperitoneally, while the *fur* mutant was avirulent only when administered orally. The loss of virulence of the *fur* mutant was partially overcome by neutralizing the pH of the stomach. Surprisingly, both the *atp* and *fur S. typhimurium* mutants were fully capable of invading and surviving within macrophages and epithelial cells. While difficult to interpret, these results suggest that the *atp* gene may play a role in virulence beyond survival of the stomach pH.

A search for *S. typhimurium* transposon mutants that are unable to survive within cultured macrophages yielded 83 mutants that have a reduced capacity

to survive the intracellular environment of murine macrophages (122). Each of these strains is less virulent for mice than is the *S. typhimurium* parent strain. These mutants have been partially characterized using assays for several different properties believed to be important in virulence. These tests revealed that 12 of the mutants are nutritional auxotrophs and 8 are nonmotile. Thirteen of the mutants have surface alterations, 9 have altered colony morphologies, 3 are hypersensitive to serum, and 1 has an altered LPS structure. Three other mutants had increased sensitivity to oxidative stress, and one Tn10 insertion was in the *S. typhimurium* virulence plasmid. No phenotype was reported for the other 46 macrophage-sensitive mutants. A follow-up report has partially characterized 30 more of these macrophage-sensitive mutants (129). Physical mapping of these transposon insertions has revealed that they were widely dispersed around the chromosome, which suggests that the bacterial survival mechanism is both complex and multifaceted. Some of the transposons had inserted into known genes; however, the majority of the transposons were in previously unstudied loci on the Salmonella chromosome. Three of these mutants were selected for analysis based on the observations that they had lost the ability to persist in mice for even short periods of time and were unable to elicit any significant humoral or cellular immune response.

As an initial characterization step, the effect of crude phagocytic cell extracts on the mutants was tested. Such extracts were found to have a strong microbiocidal effect on each of these three strains, but not on other macrophage-sensitive mutants. Subsequently, it was demonstrated that small cationic peptides known as defensins were responsible for the killing activity of the crude cellular extracts. These antimicrobial peptides are present in large quantities in the granules of macrophages and neutrophils and are believed to function by forming pores in bacterial membranes (130). Transductional mapping studies indicated that each of the insertions were located at 25 minutes on the Salmonella chromosome where three genes, *purB, phoP,* and *pepT,* are known to reside. The results of several experiments demonstrated that the mutants that were hypersensitive to defensins had insertions in the *phoP* locus, which is known to regulate the acid phosphatase gene *phoN* in phosphate starvation conditions (131). Groisman et al demonstrated that the lack of this phosphatase was not responsible for defensin sensitivity and avirulence, since phoN mutants were fully virulent and had wild-type levels of resistance to defensins. The *phoP* gene was proposed to be a regulator of multiple Salmonella genes, of which at least one is required for virulence.

As a result of this work, a search was conducted for genetic loci involved in conferring resistance to antimicrobial peptides (132). A screen of a bank of MudJ transposon insertions identified defensin-sensitive mutants mapped

to several distinct locations on the Salmonella chromosome. One group of insertions that mapped to 33 minutes on the chromosome was selected for detailed analysis (133). To gain information about the virulence factors at 33 minutes, the genes were cloned by complementation of the defensin-sensitive mutant and sequenced to gain information about possible functions. Five open reading frames, designated *sapA, -B, -C, -D, -F,* were found arranged as an operon. The *sapA* gene sequence displayed identity with periplasmic solute binding proteins involved in peptide transport. Two other genes, *sapD* and *sapF,* had sequence similarity to the "ATP-binding" cassette family of transporters, which are involved in the transport of oligopeptides, the export of yeast peptide mating pheromones, and resistance to chemotherapy agents. One possible mechanism for defensin-resistance mediated by this *S. typhimurium* gene cluster is importation of antimicrobial peptides into the cytoplasm of the bacteria to prevent pore formation in the membranes. The defensins in the cytoplasm could then be degraded by proteases.

Many groups have reported that the *S. typhimurium phoP* gene is important in Salmonella pathogenesis (120, 121, 134). Miller et al (120) were the first to demonstrate that the *phoP* locus was comprised of two genes, *phoP* and *phoQ,* which are highly similar to other bacterial regulators that respond to environmental signals. A search for genes controlled by the *phoP/phoQ* system was conducted, and three *lacZ* fusion mutants were obtained that required *phoP/phoQ* for efficient expression of β-galactosidase. The *S. typhimurium* strain carrying one of these mutations, termed *pagC1,* is less virulent for mice, while the virulence of the other two mutants is unaffected by *lacZ* transposon insertions. The *pagC* gene was cloned, sequenced, and shown to "rescue" the virulence defect of the *S. typhimurium pagC* mutant. While the function of this 18-kDa *pagC* gene product remains undefined, it appears to be a membrane protein with extensive similarity to the *Yersinia enterocolitica* Ail protein (135), which confers serum resistance (136, 137). Recent work has provided evidence that the *pagC* gene is highly expressed in vivo (138). Mice immunized with a heterologous antigen (alkaline phosphatase), whose expression was under the control of the *pagC* promoter, had a significant antibody response to the heterologous antigen. No significant antibody response was observed from mice immunized with the same antigen when it was constitutively expressed. Expression of a fifth locus, *psiD,* which is regulated by levels of phosphate available for growth but is not required for virulence, is also under the control of *phoP* (121). Collectively, these results indicate that the *phoP/phoQ* regulatory system positively regulates the expression of many genes, including virulence determinants with unknown activities. In addition, this global regulator appears to control resistance to antimicrobial peptides found within professional phagocytes.

In addition to inducing the expression of Salmonella genes, the *phoP/phoQ* regulon also represses the expression of many genes (139). Behlau & Miller (114) have identified and examined some of the genes repressed by this global regulatory system. They obtained mutants in five separate loci, using *phoA* operon fusions, which are significantly repressed by the conditions (starvation and low pH) that induce *phoP*-activated genes. Only one of these genes, *prgH*, was found to contribute to mouse virulence, and virulence was attenuated by either the oral or intraperitoneal route of infection. However, the loss of virulence could not be attributed to an intracellular survival defect in macrophages, as the *prgH* mutant survived as well as wild type *S. typhimurium* in macrophages. In addition, as discussed above, this *prgH* mutant has decreased levels of invasiveness as measured with a tissue culture invasion assay. These results indicate that the *phoP/phoQ* regulatory system both positively and negatively regulates the expression of several genes, including factors that play a role in invasion and virulence.

Leung & Finlay (140) have also identified genes necessary for intracellular replication and virulence. Three separate prototrophic mutants have been obtained that are avirulent for mice by both oral and intraperitoneal routes of infection. These strains are fully invasive but are unable to replicate in a variety of tissue culture cells. Presumably, the mutants do not replicate within phagocytic cells during infection. An understanding of these genes and their contribution to macrophage intracellular survival and virulence awaits the isolation and analysis of each gene.

In the broader context of bacterial pathogenicity, it can be seen that Salmonella, like all other invasive microbes, face a potential barrage of phagocytic cells and innate host defense mechanisms once past the epithelial barrier of the host. The successful pathogen does not ordinarily dare to incite the host too quickly; its primary purpose is to replicate sufficiently to ensure its perpetuation before taking on the full force of host defense factors. This is not always the case. Shigella appears to deliberately inflame the host defense mechanisms. The resulting influx of inflammatory cells into the bowel epithelium exposes the basolateral aspect of many cells, so that the bacteria which have replicated in the lumen can better invade more enterocytes. Of course, Shigella has the added advantage that it rarely needs to establish long-term carriage. Thus, while the shared features of attachment and entry for enteric pathogens are obvious from genetic and molecular analysis, after entry the bacterial strategies for replication appear to be different. Salmonella seem to have learned to exploit the intracellular trafficking pathways of host cells to become lodged in a privileged niche. Initially, Shigella probably enter the same intracellular niche as Salmonella, but quickly exit to the nutrient-rich, nonthreatening cytoplasm

of the cell. It is also important to see that the initial phase of interaction after passage through the epithelial barrier seems to be with resident phagocytic and other antigen-presenting cells.

Every invasive pathogen must learn to deal with this phagocytic cell hurdle. Some Gram positive microbes simply poison the phagocytes. Shigella causes the macrophage to commit cellular suicide by apoptosis. Salmonella boldly enter the macrophage with a ruffle and have the temerity to replicate there and kill the cell. We believe that Salmonella goes undetected by phagocytes and the inflammatory cascades in the early stages of host-adapted infection, possibly because the vacuole in which it is sequestered has been cleverly modified. We currently know less about the genetic and molecular basis of intracellular life than we do about bacterial entry into cells. However, the genetic tools are at hand, and the methods of the cell biologist and the immunologist are being employed increasingly to examine this pivotal aspect in the pathogenesis of infection.

9. THE SALMONELLA VIRULENCE PLASMID AND SYSTEMIC INFECTION

Many serovars of Salmonella carry large plasmids (50–90 kb) which are associated with virulence (141). Early work suggested that these plasmids carried invasion-related genes (142), but it was subsequently demonstrated that the plasmid was required not for cell entry but for persistence within the RES (143). A highly conserved region of these plasmids contains five genes, designated *spvRABCD*, on 8 kb of DNA, which are able to restore wild-type levels of virulence to plasmid-cured strains (144–146). As for other Salmonella virulence genes, expression of the plasmid virulence genes is regulated by environmental conditions. Sequence analysis of *spvR* found that *SpvR* (147) was a member of the LysR family of positive regulatory elements (148). Several groups have demonstrated that SpvR positively regulates the expression of some or all of the *spv* genes (149–152). This induction is highest when bacterial cultures enter the stationary or starvation phase of growth (150). Work by Fang et al (153) demonstrated that the *katF* gene, which encodes a stationary phase–specific alternative sigma factor, is required for maximal expression of *spvABCD*. As expected from these results, a *katF S. typhimurium* mutant is attenuated for virulence in mice.

Two observations suggest possible functions for the *spvR, -A, -B, -C, -D* plasmid genes. Intraperitoneal injection of plasmid-cured *S. typhimurium* into mice elicited recruitment of $\gamma\delta$ T cells, while the wild-type strain did not (154). These T cells, which predominantly express Vγ1/Vδ6 T cell receptors,

are associated with responses to heat shock proteins. Gulig & Doyle recently demonstrated that the presence of the virulence plasmid increases the growth rate of *S. typhimurium* within the RES of mice (155). Perhaps the role of $\gamma\delta$ T cells is to prevent the growth of bacteria in the host while the *spvRABCD* gene products allow pathogenic Salmonella species to suppress the $\gamma\delta$ T cell response.

10. CONCLUSION

The focus of our review has been the bacterial strategy to prevail over the host immune system. We have paid particular attention to the molecular and genetic basis of Salmonella pathogenicity. We have broadened our perspective, where appropriate, to discuss the attributes of other pathogenic microorganisms to unify some general concepts of bacterial pathogenesis. In all scientific disciplines, the experiments of nature usually prove to be the most instructive if we are attentive. Certainly, immunologists have learned a great deal about the intricacies of the immune system from individuals with various immunodeficiencies. Thus, the kinds of infections suffered by individuals with chronic granulomatous disease (CGD) reveal insights into the functioning of our immune defenses; they also help the bacteriologist identify bacterial factors that allow specific organisms to opportunistically exploit those with CGD. There is another facet of this approach that has not been adequately exploited in the study of the immune system. Defined bacterial mutants that are deficient in their capacity to overcome immune defenses can be of great use in probing the nature of anti-bacterial immunity. LPS is not a substitute for a whole, living microorganism. All pathogens are not alike, nor are they all facultative intracellular parasites like Salmonella. Microbiologists for their part tend to ignore the whole animal for the simulated growth in laboratory medium or within the confines of the tissue culture flask. We only believe that we know what cell types are actually encountered by pathogens in the host. Much of what we believe is based on conventional wisdom gleaned from the pathology of terminally ill animals (and genetically susceptible ones at that), necropsy findings, and only rarely on biopsy material.

The view of bacterial pathogenicity based on disease is really an amalgam of microbial action and host response—mostly host response, if one is a clinician. We believe it is rather more important to understand fully the parameters of infection and the early interplay between the microorganism and its host. It is easy to overlook the fact that both we and the microbes that infect us are still in the process of evolving. The bacteria will do it far better and faster than we can. It represents a challenge to our health as in the case of "diseases of human progress" like AIDS and the resurgence of food-borne salmonellosis.

Scientifically, however, it represents an opportunity to exploit the microorganism as a research tool to examine the intricacies of the immune system. Microorganisms after all may be the most knowledgeable immunologists of all.

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THE STRUCTURE OF THE T CELL ANTIGEN RECEPTOR

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ABSTRACT

Recent crystallographic studies of T cell antigen receptor (TCR) fragments from the α and β chains have now confirmed the expected structural similarity to corresponding immunoglobulin domains. Although the three-dimensional structure of a complete TCR $\alpha\beta$ heterodimer has not yet been determined, these results support the view that the extracellular region should resemble an immunoglobulin Fab fragment with the antigen-binding site formed from peptide loops homologous to immunoglobulin complementarity-determining regions (CDR). These preliminary results suggest that CDR1 and CDR2 may be less variable in structure than their immunoglobulin counterparts, consistent with the idea that they may interact preferentially with the less polymorphic regions of the molecules of the major histocompatibility complex. The region on the variable β domain responsible for superantigen recognition is analyzed in detail. The implications for T cell activation from the interactions observed between domains of the α and β chains are also discussed in terms of possible dimerization and allosteric mechanisms.

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1. INTRODUCTION

The primary event in the cellular immune response is recognition of the antigen by membrane-bound T cell antigen receptors (TCR). The TCR is composed of a glycosylated polymorphic heterodimer, either $\alpha\beta$ or $\gamma\delta$, which is associated with a nonpolymorphic membrane-bound complex of proteins collectively known as CD3 (1). Each polypeptide chain of the heterodimer is, for the most part, located on the extracellular side of the membrane, with a hydrophobic region positioned toward the C terminus, which makes a single passage across the lipid to finish with a short cytoplasmic tail of 2-7 amino acids (2). The two polypeptide chains are linked by a cystine bridge in the extracellular region close to the membrane of the T cell. The $\alpha\beta$ form of heterodimer recognizes antigen in the form of peptide degradation products. T cell recognition is restricted in this case to those peptides bound to molecules of the major histocompatibility complex (MHC), class I and II, which are membrane-bound proteins displayed on the surface of antigen-presenting cells (APC) (3). Antigen recognition by $\gamma\delta$ TCR is more diverse and is generally less well characterized (4). This type of heterodimer can, like the $\alpha\beta$ receptor, recognize antigen bound to MHC and related molecules; other $\gamma\delta$ receptors respond to carbohydrate- or nucleotide-carrying antigen for which the mode of presentation is poorly characterized.

Although much detail is known of $\alpha\beta$ TCR recognition at the level of three-dimensional structure, certain key aspects still await elucidation. At present, the structure of several class I MHC molecules and one class II MHC molecule have shown how antigenic peptide is bound and displayed by presenting molecules (5). Although the structure of a complete TCR has not yet been reported, homology in amino acid sequence strongly suggests a close relationship to the structure of immunoglobulins. By contrast with immunoglobulins, progress toward describing the structure of the TCR by X-ray diffraction techniques has been slow. Problems of expression of soluble TCR in quantity and the natural presence of carbohydrate on the molecule have presented difficulties in realizing the essential step of crystallization. The crystallographic analysis of the β chain of the 14.3.d clone (6) [specific for a hemagglutinin peptide of influenza virus A/PR/34 (H1N1) in association with the MHC class II I-E^d molecule] has now given a first view of TCR fragments in three-dimensions, confirming a structural similarity between T cell and B cell receptors. In this review, we compare TCR structure, provided by the 14.3.d β chain result, with that of immunoglobulins, and we discuss its implications for MHC/peptide and superantigen (SAg) recognition. We also discuss briefly the Vα domain from the 1934.4 clone (7) (specific for the N-terminal nonapeptide of myelin basic protein in association with the MHC class II I-A^u molecule) and the initiation of signal transduction in the light of these structures.

2. STRUCTURE OF A TCR β CHAIN

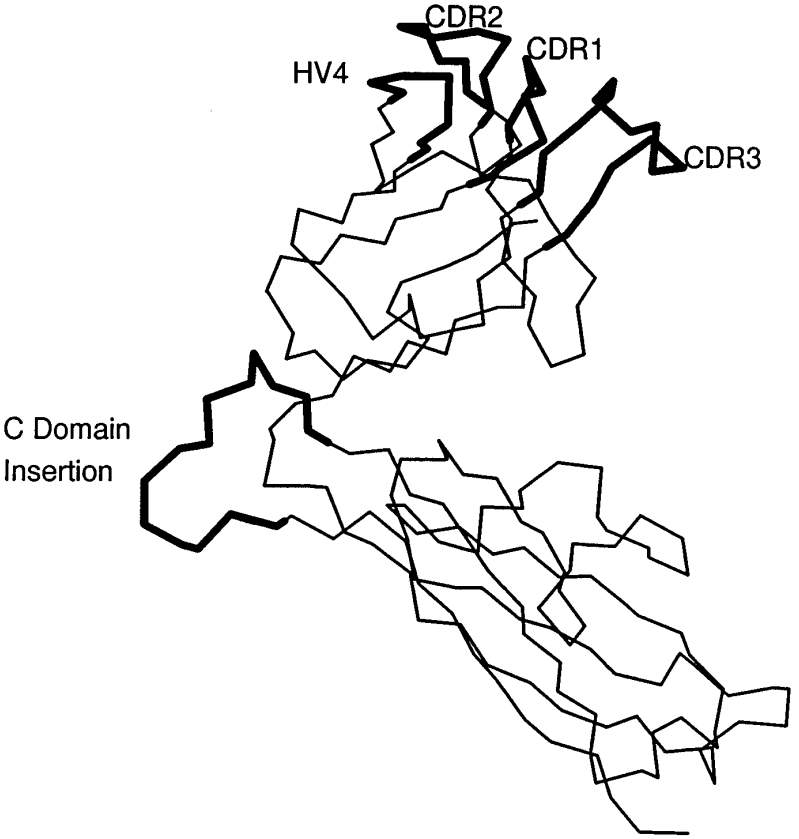
2.1 *Structure of the Domains*

The TCR β chain of the clone 14.3.d is composed of two domains that in their three-dimensional structure closely resemble the variable and constant domains of immunoglobulins (Figure 1*a*). The variable domain of the β chain (Vβ), coded by the Vβ8.2 and 2Jβ2.1 genes, bears a closer resemblance to V_L than to V_H in the framework regions. Thus, a comparison of 86 framework residues of a sample of nine different murine V_L domains (Figure 2*a*) shows a root mean square (r.m.s.) deviation of 1.4 å in α-carbon positions, while a similar comparison of 87 framework residues from nine murine V_H domains (Figure 2*b*) shows a r.m.s. deviation of 1.9 å. The largest departure from immunoglobulin variable domain framework structure occurs in the connecting loop between β strands C″ and D (see 8 for strand nomenclature), which is distant from the putative antigen-binding site of the TCR. Although the polypeptide chains of V_L and V_H are similar in this region, the conformation between Gly58 and Gly63 of Vβ8.2 is significantly different. The loops of Vβ that form the expected antigen-binding site are similarly placed as the complementarity-determining regions (CDR) of immunoglobulins; these are discussed in detail below.

The constant domain, coded by the Cβ1 gene, is similar to immunoglobulin constant domains. A structural comparison with C_H1, C_H2, C_H3, and Cκ gives a r.m.s. deviation in α-carbon positions for aligned structures of 3.0 å, 2.3 å, 2.3 å, and 2.7 å, respectively, for the 89 equivalent residues. By comparison, the r.m.s. deviation in the same α-carbon positions between different immunoglobulin constant domains varies between 1.7 å and 2.3 å. The most notable difference between the Cβ1 domain and immunoglobulin constant domains is the presence of a large insertion comprised of residues 219 to 232, inclusive (an insertion of 14 amino acids with respect to C_H1, C_H2, and Cκ, and 13 amino acids with respect to C_H3), in the loop connecting the last two strands (G and F) of the constant domain (Figure 1*a*). This loop is exposed to the solvent, making few direct contacts, either intra- or intermolecular. A second region differing significantly from the immunoglobulin constant domain folding occurs between residues 133 to 144, inclusive, and can probably be attributed to the absence of the α chain in the crystal structure; a closer resemblance here would be expected in the heterodimer.

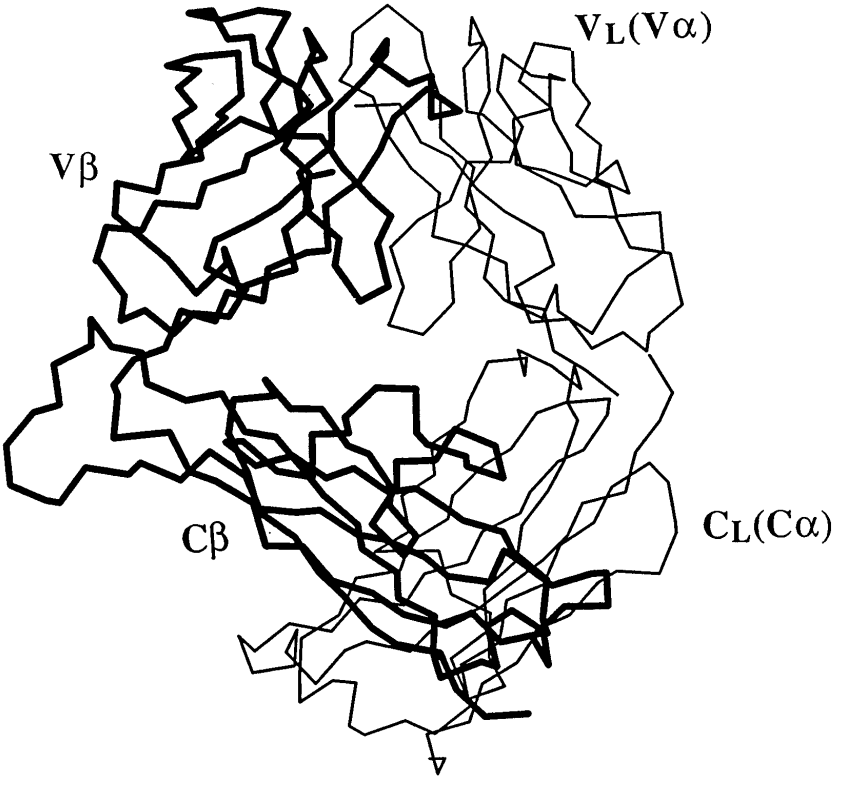
2.2 *The Complementarity-Determining Regions*

The antigen-binding site of the T cell receptor has been predicted to consist of regions analogous to the CDRs of immunoglobulins (9). While no direct structural data on TCR/MHC/peptide interactions are available at present, several studies involving mutagenesis of the receptor have implicated these regions in



(a)

Figure 1 The structure of the β chain of the 14.3.d clone. (*a*) An α-carbon skeleton of the β chain, highlighting in bold line the complementarity-determining regions CDR1, CDR2, and CDR3, the fourth hypervariable region HV4, and the loop between residues 219 and 232 in the Cβ domain, which corresponds to an insertion with respect to immunoglobulin constant domains. (*b*) A model of the TCR based on the β chain of the 14.3.d clone (*bold line*) and immunoglobulin structure. Domains V_L and C_L were placed with respect to Vβ and Cβ, respectively, to give variable and constant dimer interfaces similar to those found in immunoglobulin structures. Only α-carbon positions are shown.



(b)

Figure 1 (*continued*)

interactions with the MHC/peptide antigen (10–15). In the crystal structure of the β chain from the 14.3.d hybridoma, the CDRs are arranged similarly to those of immunoglobulins. These present some interesting comparisons with immunoglobulin CDR structure, in particular with respect to the existence of canonical conformations as found in immunoglobulins (16, 17).

The conformation of CDR1 in the β chain is stabilized by its interaction with residue Gln25 [Kabat numbering (18) is used throughout], buried within the interior of the domain beneath the hypervariable loop (Figure 3). Both the oxygen and the nitrogen of the side-chain amide group of Gln25 form a total of three hydrogen bonds to main-chain atoms of CDR1, thus providing a supporting scaffold for the loop as it passes from one β sheet to the other. This contrasts with immunoglobulins where the structure of CDR1 in both V_H and V_L is stabilized by intercalation of an apolar residue at position 29 between the

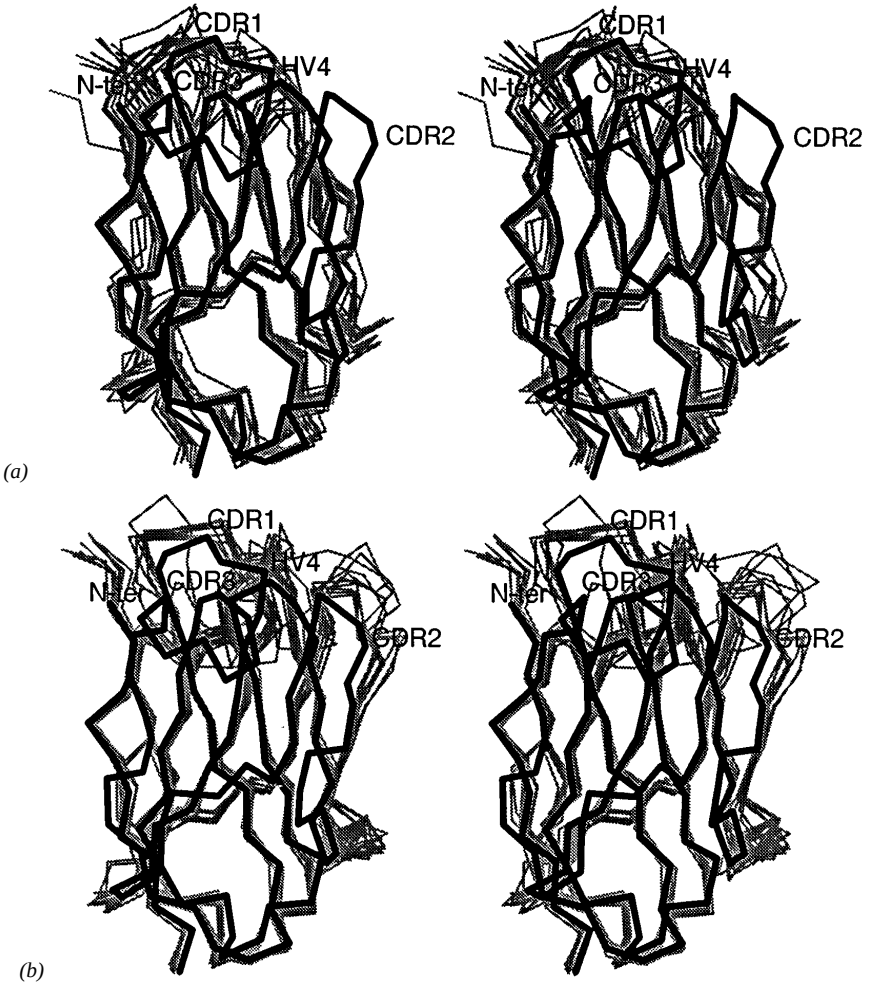


Figure 2 Structural comparison of the Vβ domain of the 14.3.d clone. (*a*) Stereo view of Vβ (*solid line*) superimposed on nine murine V_L domains (*grey line*) (r.m.s. difference of 1.4 å in α-carbon positions of framework residues). (*b*) Stereo view of Vβ (*solid line*) superimposed on nine murine V_H domains (*grey line*) (r.m.s. difference of 1.9 å in α-carbon positions of framework residues). The amino terminus is marked as N-ter, the three complementarity-determining regions as CDR1, CDR2, and CDR3, and the fourth hypervariable region as HV4.

two β sheets of the framework structure, occupying a hydrophobic environment (16, 17). [For murine V_{kappa}, the Kabat numbering (18) is 27B, 28, or 30 in some subgroups but is always four residues away from position 25. For murine V_{λ}, this corresponds to residue 27C and is 5 residues from position 25.] Residue 29 is located approximately midway along the immunoglobulin CDR1, forming an optimum anchorage point to stabilize the conformation of this loop. In murine immunoglobulin sequences (18), residue 29 is most often Phe, Leu, Ile, or Val. (A very small fraction of sequences occurs in which this residue is polar, but there are no structural data for these rare instances.) In Vβ8.2, however, the side chain of Gln25 occupies the volume normally taken by the side chain of residue 29 in V_L, or residues 27 and 29 in V_H, and His29 of the β chain is displaced sideways, becoming partially exposed to the solvent. Thus, a different structural solution is used to maintain stability in the Vβ8.2 CDR1 to that found in either the heavy or light chain immunoglobulin hypervariable region. Immunoglobulin residues homologous to Gln25 of Vβ8.2 (residue 25 in V_L and residue 24 in V_H) are usually small in volume (most frequently Gly, Ala, or Ser, and less often, Val or Thr), probably in compliance with the volume taken by the buried hydrophobic side chain of residue 29 (and residue 27 in

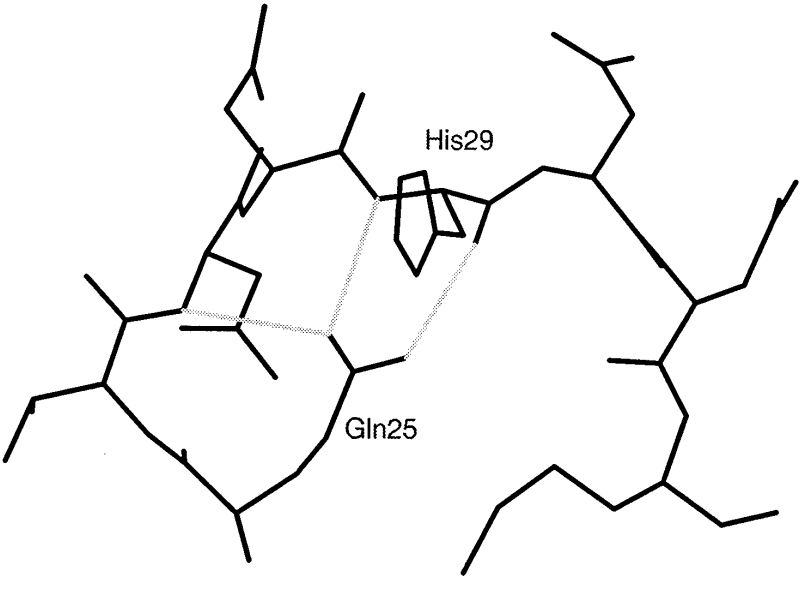


Figure 3 The CDR1 loop between residues Gln25 and Met32. Hydrogen bonds from Gln25 to other residues in CDR1 are indicated in grey line.

V_H). In very few exceptions, larger side chains such as Tyr, Phe, Leu, and Ile are to be found in position 25 (or 24 for V_H) of immunoglobulin variable domains, but there are no three-dimensional data to show the consequences for these examples.

The CDR1 of Vβ are less variable in length than immunoglobulin variable domains: 19 murine Vβ domains have 10 amino acids between the conserved residues Cys23 and Trp34, and 3 have 11 residues (Table 1). In murine immunoglobulins, this can vary from 11 to 17 amino acids for V_L and from 13 to 15 amino acids for V_H. Based on the criteria of a common loop length and the presence of Gln at the key position 25, the conformation of CDR1 observed in the crystal structure of the 14.3.d β chain might describe a canonical structure present in a total of nine murine Vβ domains: Vβ1, Vβ4, Vβ6, Vβ7, Vβ8.1, Vβ8.2 (which codes for 14.3.d), Vβ8.3, Vβ9, and Vβ10.

Each of these Vβ domains has, in addition, His at position 29, but it remains unclear if this residue plays a critical role in determining the main-chain conformation of CDR1. We note, however, that Nϵ2 of His29 forms a hydrogen bond to Oγ of Ser94 in the crystal structure, a residue common to all these domains with the exception of Vβ7. Interestingly, all Vβ domains with Pro at position 25 (Vβ3, Vβ5.1, Vβ5.2, Vβ11, Vβ16, Vβ17, Vβ19, and Vβ20) also have His29 and Ser94 as conserved residues, but without structural data on at least one of these domains, it is not clear what significance this observation may have. We may conclude, nonetheless, that Vβ domains with Pro at position 25 will be different in structure from Vβ8.2 at CDR1 because the restricted conformational freedom of Pro is not compatible with the main-chain conformation of Gln25 = (-126°,154°) for Gln25, while ϕ must be close to -60° for Pro. It is remarkable, however, that His29 occurs in 17 Vβ domains. Such a high frequency might conceivably be related to requirements imposed by antigen recognition, such as defining specific MHC-TCR contacts.

Finally, we add a note of caution that Vβ1, Vβ4, and Vβ10 may differ from the Vβ8.2 structure, at least in detail. Since these have Gly at position 28, allowing a greater degree of conformational freedom, and, in addition, none have Arg at position 69, which forms hydrogen bonds to the main chain of Vβ8.2 CDR1, we cannot exclude the possibility of a difference in structure, even if Gln25 stabilizes the loop in a similar manner. Whether CDR1 of Vβ8.2 does indeed describe a canonical form for as many as nine murine Vβ genes will require further investigation.

The CDR2 of Vβ8.2 is four residues longer than the homologous hypervariable region of V_L and is thus closer to that of V_H. It forms a seven-residue loop containing a type II β turn between residues 51 and 54. In addition, the side chain of Ser49 forms three hydrogen bonds to main chain atoms within the

Table 1

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                 1         2         3         4         5         6         7         8         9
        123456789012345678901234567890A123456789012345678901234567890123456789012A345678901234567890123456789012345678901234

Vβ1     NTKITQSPRYLILGR-ANKSLECEQHLGHN-AMYWYKQSAEKPPELMFLYNLKQLIRNETVPS--RFIPECPDSSKLLLHISAVDPEDSAVYFCAS
Vβ2      TLLEQNPRWRLVPRGQAVNLRCILKNSQYPWMSWYQQDLQKQLQWLFTLRSPRDKEVKSLPG-ADYLATRVTDTELRLQVANMSQGRTLYCTCSA
Vβ3     NSKVIQTPRYLVKGQGQKAKMRCIPEKGHP-VVFWYQQNKNNEFKFLINFQNQEVLQQIDMTE-KRFSAECPSNSPCSLEIQSSEAGDSALYLCAS
Vβ4     DPKIIQKPKYLVAVTGSEKILICEQYLGHN-AMYWYRQSAKKPLEFMFSYSYQKLMDNQTASS--RFQPQSSKKNHLDLQITALKPDDSATYFCAS
Vβ5.1   NSGVVQSPRYIIKGKGERSILKCIPISGHL-SVAWYQQTQGQELKFFIQHYDKMERDKGNLPS--RFSVQQFDDYHSEMNMSALELEDSAVYFCAS
Vβ5.2   DSGVVQSPRHIIKEKGGRSVLTCIPISGHS-NVVWYQQTLGKELKFLIQHYEKVERDKGFLPS--RFSVQQFDDYHSEMNMSALELEDSAMYFCAS
Vβ6     GGIITQTPKFLIGQEGQKLTLKCQQNFNHD-TMYWYRQDSGKGLRLIYYSITENDLQKGDLSE-G-YDASREKKSSFSLTVTSAQKNEMAVFLCAS
Vβ7     DMKVTQMSRYLIKRMGENVLLECGQDMSHE-TMYWYRQDPGLGLQLIYISYDVDSNSEGDIPK-G-YRVSRKKREHFSLILDSAKTNQTSVYFCAQ
Vβ8.1   EAAVTQSPRSKVAVTGGKVTLSCHQTNNHD-YMYWYRQDTGHGLRLIHYSYVADSTEKGDIPD-G-YKASRPSQENFSLILELASLSQTAVYFCAS
Vβ8.2   EAAVTQSPRNKVAVTGGKVTLSCNQTNNHN-NMYWYRQDTGHGLRLIHYSYGAGSTEKGDIPD-G-YKASRPSQENFSLILELATPSQTSVYFCAS
Vβ8.3   EAAVTQSPRNKVTVTGGNVTLSCRQTNSHN-YMYWYRQDTGHGLRLIHYSYGAGNLRIGDVPD-G-YKATRTTQEDFFLLLELASPSQTSLYFCAS
Vβ9     DTTVKQNPRYKLARVGKPVNLICSQTMNHD-TMYWYQKKPNQAPKLLLFYYDKILNREADTFE--KFQSSRPNNSFCSLYIGSAGLEYSAMYLCAS
Vβ10    ETAVFQTPNYHVTQVGNEVSFNCKQTLGHD-TMYWYKQDSKKLLKIMFSYNNKQLIVNETVPR--RFSPQSSDKAHLNLRIKSVEPEDSAVYLCAS
Vβ11    NAGVIQTPRHKVTGKGQEATLWCEPISGHS-AVFWYRQTIVQGLEFLTYFRNQAPIDDSGMPK-ERFSAQMPNQSHSTLKIQSTQPQDSAVYLCAS
Vβ12                                                            FPLMEDGGAFKDRFKAEMLNSSFSTLKIQPTEPKDSAVYLCAS
Vβ13    KLESPSLPDMQSYAMGKLFPFGVTLFLDID-TLYWYQQPRDQGPQLLVYFADEAVIDNSQLPS-DRFSAVRPKGTNSTLKIQSAKQGDTATYLCAS
Vβ14    AQTIHQWPVAEIKAVGSPLSLGCTIKGKSSPNLYWYWQATGGTLQQLFYSITVGQVESVVQLN---LSASRPKDDQFILSTEKLLLSHSGFYLCAW
Vβ15    GALVYQYPRRTICKSGTSMRMECQAVGFQATSVAWYRQSPQKAFELIALSTVNSAIKYEQNFTQEKFPISHPNLSFSSMTVLNAYLEDRGLYLCGA
Vβ16    GPKVLQIPSHQIIDMGQMVTLNCDPVSNHL-YFYWYKQILGQQMEFLVNFYNVKVMEKSKLFK-DQFSVERPDGSYFTLKIQPTALEDSAVYFCAS
Vβ17      GVTQTPRYLVKEKGQKAHMSCSPEKGHT-AFYWYQQNQKQELTFLINFRNEEIMEQTDLVK-KRFSAKCSSNSQCILEILSSEEDDSALYLCAS
Vβ18    SVLLYQKPNRDICQSGTSLKIQCVADSQVV-SMFWYQQFQEQSLMLMATANEGSEATYESGFTKDKFPISRPNLTFSTLTVNNARPGDSSIYFCSS
Vβ19    DSGVVQNPRHLVKGKEQKAKMDCTPINGHS-YVYWYYKKPGEELKFLVYFQNEDIIDKIDMIG-KNISAKCPAKKPCTIEIQSSKLTDSAVYFCAS
Vβ20    DAAVTQKPRYLIKMKGQEAEMKCIPEKGHT-AVFWYQQKQSKELKFLIYFQNQQPLDQIDMVK-ERFSAVCPSSSLCSLGIRTCEAEDSALYLCSS
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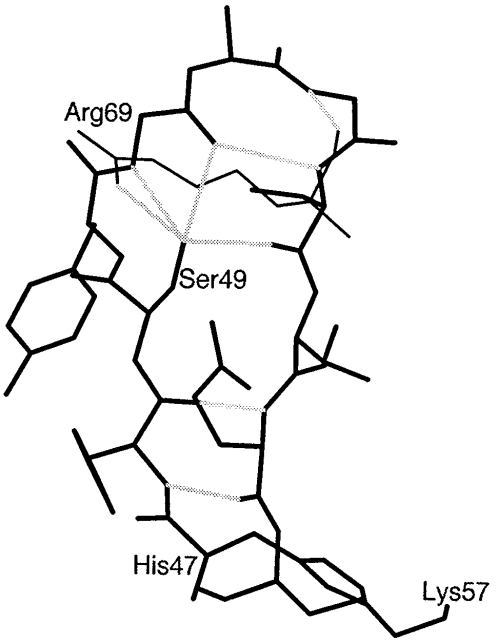

Figure 4 The CDR2 loop between residues His47 and Lys57. The framework residue Arg69 is shown behind CDR2 in thin line. Hydrogen bonds are indicated in grey line.

loop, and a hydrogen bond is formed between Oγ of Ser54 and the carbonyl oxygen of Gly51 (Figure 4). The framework residue Arg69 exerts an additional influence on the conformation of the loop; the guanidyl group forms a hydrogen bond to Oγ of Ser49, and its carbonyl group forms a hydrogen bond to the main chain at residue 53. By homology, Arg69 corresponds to position 71 in V_H, which also is often an arginine residue. The influence of Arg at position 71 in V_H upon the conformation of CDR2 has been already noted (19).

This rather complex constellation of secondary structural elements contributing to the conformation of CDR2 in Vβ8.2 may be repeated in other Vβ domains and may thus represent a canonical structure for this TCR hypervariable region. We suggest that the key features determining the conformation of this seven-residue loop are (i) the presence of Ser at position 49, (ii) the ability to form a type II β turn between residues 51 and 54, and (iii) the presence of Arg at position 69. The presence of a type II β turn is less straightforward to assess from the sequence alone. Type II β turns are favored by Gly at the third residue of the turn because steric hindrance between the carbonyl group of the second residue

and the side chain of the third leads to an unfavorable energy contribution to the conformation (20). While Gly appears most frequently at this position for type II β turn, Asn occurs with significant probability as well (21). Nonetheless, other amino acids can also appear here, suggesting that interactions with neighboring regions of the protein can compensate for the unfavorable energy contribution. The domains Vβ6, Vβ7, Vβ8.1, Vβ8.2, Vβ8.3, and Vβ14 are of equal length in their CDR2, and all have Ser49 and Arg69 as common residues. Only Vβ6, Vβ8.2, Vβ8.3, and Vβ14, however, have the favorable Gly or Asn at residue 53 that would allow us to predict the presence of the required type II β turn with confidence. The hydrogen bond formed between main chain atoms of residues 53 and Arg69, however, may provide the necessary stabilization to favor a type II β turn in Vβ7 and Vβ8.1 as well. Consistent with this possibility, DiGuisto & Palmer (22) found no loss in antigen recognition upon introducing the mutation Asp53Gly into the Vβ8.1 domain of a T cell hybridoma. Thus, the CDR2 conformation found in the 14.3.d β chain may be common to at least four and perhaps as many as six Vβ domains.

The CDR3 of the 14.3.d β chain is longer than the corresponding V_L hypervariable loop by an average of about four residues and is thus more comparable in this respect to CDR3 of V_H. As with V_H, the CDR3 of Vβ is also coded by D and J gene segments. It is therefore interesting that this hypervariable loop in the 14.3.d β chain also shares the following structural characteristic generally found in the CDR3 of V_H. In all reported structures, CDR3 of V_L folds back over the surface of the domain toward CDR1 and CDR2. By contrast, CDR3 of V_H folds away from the surface of the domain and is thus directed toward CDR3 of V_L of the accompanying chain. [Two exceptions are the mAb 8F5 (23) and HyHEL-5 (24) where the loop of V_H CDR3 folds in a way similar to V_L.] In this respect, the β-chain CDR3 resembles the V_H CDR3 because it also is directed away from CDR1 and CDR2. The region of CDR3 of 14.3.d coded by the diversity region is glycine-rich; this residue is present at positions 95, 96, 97, and 99. The high frequency of Gly in CDR3 of TCRs (and immunoglobulin heavy) has been noted by Abergal & Claverie (25), who suggested that this unusual occurrence may arise to meet conformational constraints particular to CDR3 of V_H. Since Dβ gene segments can be read in any reading frame, a predominance of polyguanine stretches in TCR Dβ genes allows Gly to be transcribed with high probability.

As in immunoglobulins, an additional β-hairpin loop occurs adjacent to the CDR loops of the variable domain of the 14.3.d β chain. This region is homologous to the third framework region (FR3) of immunoglobulins and is often referred to as the fourth hypervariable region of the TCR, since a hypervariability has been demonstrated in this part of the TCR sequence (26).

The V_L and V_H domains differ in their FR3 region. In the V_L domain, the FR3 loop folds in toward CDR1 and CDR2, making direct atomic contacts with both, while in the V_H domain it is almost always directed away toward the solvent. [V_H of 4-4-20 (27) is an exception.] Furthermore, in comparison to V_H, the FR3 loop of V_L is shorter by two residues. In the β chain crystal structure, the region of Vβ8.2 corresponding to FR3 folds toward CDR1 and CDR2 and is thus closer to V_L. In length, however, the loop falls intermediate between that of V_L and V_H. Should the conformation of the loop observed in Vβ8.2 be adopted more generally in other Vβ (or Vα) domains, it would indeed be more favorably placed to contact the MHC-peptide complex than if it were like V_H in conformation. The idiotope-anti-idiotope complex FabD1.3-FabE225 (28) offers an example in which antibody-antigen contacts are mediated by residues in the FR3 region. In this structure, some FR3 residues of the V_L domain of each antibody fragment make contacts with the other component of the complex. In the light of the β-chain structure, it is therefore conceivable that contacts made by the HV4 loop may also have a role in T cell immune recognition (29).

2.3 *Quaternary Organization of the β-Chain*

The numerous structural studies of Fab fragments have revealed an intrinsic flexibility in the region connecting the variable and constant domains of both the light and heavy chains. Thus, while the association between V_H and V_L and between C_L and C_H1 remains relatively invariant for different Fab structures, the elbow angle (defined by the angle subtended by the approximate twofold axes relating V_H to V_L and C_H1 to C_L) takes values ranging between 127° and 193° (30). The variation in relative orientation between the variable and constant dimers of Fabs manifests itself as a movement pivoting on a conserved set of interactions between a small number of residues of V_H and C_H1, which are invariant or semi-invariant in sequence (31). Contacts between the light chain domains, by contrast, do not show a use of equivalent residues in different Fab structures, although the number of interdomain contacts is generally larger than for the immunoglobulin heavy chain. It has been suggested that the mode of interaction existing between the immunoglobulin heavy chain domains may also occur between the Vβ and Cβ domains, since a similar pattern of conserved or semi-conserved residues is found at equivalent positions of the TCR β-chain (9, 31). Although the residues predicted by these authors do make contacts across the Vβ/Cβ interface (Val12, Thr115, Val16A, Phe153, and Pro154), they do not appear to be the most important in the crystal structure of the 14.3.d β chain. Several van der Waals contacts are established between His156 of Cβ and residues from the variable domain, including a hydrogen bond between Nϵ2 and Oγ of Thr115. The variable domain residue Arg113 also contributes numerous nonpolar contacts across the Vβ/Cβ interface and a salt bridge to

Glu158 of Cβ. This salt bridge may be conserved since residue 113 is coded as Lys in one murine Jβ gene and as Arg in the remaining 11 Jβ genes (32).

In total, 20 residues together with three bridging solvent molecules contribute toward contacts between the two protein surfaces. The combined surface area between the two domains that is inaccessible by solvent is 833 $å^2$ (the contribution of residues 116A and 117 at the transition between the two domains was not included). Corresponding areas between V_H and C_H1 vary between about 200 $å^2$ and 350 $å^2$ depending on the elbow angle. The size of the interface between the constant and variable domains and the presence of a conserved salt bridge might suggest, in general, a more rigid structure for the β chain in comparison to immunoglobulin heavy chains.

The hydrophobic surface of the Vβ domain implicated in formation of the interface with Vα in the $\alpha\beta$ heterodimer (33) contacts the equivalent surface of a neighboring β-chain in the crystal structure, not giving an immunoglobulin Fv-like structure, but rather one where the Vβ domains pack head-to-tail so that their CDRs appear at opposing ends of the aggregate. Ten residues from each variable domain surface contribute numerous van der Waals contacts and four hydrogen bonds across this interface. These residues are Gln37, Gly42, Leu43, Arg44, Phe91, Gly99, Tyr101, Ala102, Phe107, and Gly108, some of which are equivalent by homology to those involved in the V_H/V_L interaction (34). The conformation of the CDR3 loop may be critical in determining this particular mode of association in the crystal since, as described above, it folds away from the core of the Vβ domain in a manner characteristic of immunoglobulin V_H domains. Accordingly, in the absence of any conformational rearrangement of CDR3, an immunoglobulin Fv-like homodimer formed by the Vβ domains would lead to steric encumbrance between these hypervariable loops. Taking this argument further, the Vα domain forming a heterodimer with this particular Vβ domain would itself require a V_L-like conformation in its CDR3 loop to form a Vα/Vβ Fv dimer. This is indeed the case for Vα of the 1434.4 clone (7). Whether or not this situation persists more generally in the TCR must await further structural studies.

The surface of the Cβ domain implicated in heterodimer formation is, by contrast with Vβ, completely exposed to the solvent in the crystal. This surface is rich in charged residues; a total of 5 lysines, 3 arginines, 3 glutamates, and an aspartate are located in this region or at its periphery to form an array of charges that cannot be neutralized by a complementary matching of the same surface of neighboring Cβ domain. At least three of these charged residues would be completely buried by dimer formation. This contrasts with the Cκ, Cγ, and C_H1 domains, where the dimer-forming surfaces are very hydrophobic and carry no charged residues except, in some cases, at the periphery.

Furthermore, the corresponding surface of Cα predicted to interact with Cβ in the $\alpha\beta$ heterodimer is also polar but should carry a net negative charge (33, 35). Polar residues may therefore be disposed such that charges are neutralized by salt-bridge formation across the Cα/Cβ interface in the heterodimer. In summary, therefore, the stereochemical constraints on Vβ/Vβ and Cβ/Cβ pairing discussed above would not permit formation of a β chain homodimer analogous to the immunoglobulin light-chain dimer.

2.4 *A Model for the $\alpha\beta$ Heterodimer*

Although the structure of the intact extracellular region of an $\alpha\beta$ heterodimer has not yet been reported, a model has been proposed based on the structure of the 14.3.d β chain (6). By superposition of V_H and C_H1 domains of a Fab upon the Vβ and Cβ domains, respectively, the corresponding positions of the V_L and C_L domains could be used to model the α chain without resort to any modification to the quaternary structure of the β chain to relieve steric hindrance. This model, which has the essential features present in earlier propositions based on immunoglobulin structure (9, 36, 37), is structurally equivalent to a Fab fragment with an elbow angle of 154° (Figure 1*b*). The rigid quaternary structure of the β chain suggested by the crystal structure may thus, by contrast to Fab structure, confer a relatively rigid arrangement between the variable and constant domains in the $\alpha\beta$ heterodimer. The relatively compact structure of the β chain is consistent with its remarkable resistance to a variety of proteases (papain, bromelain, trypsin, chymotrypsin) whether or not it is paired to the α chain (38). By contrast, the α chain is readily digested by these enzymes, even when paired with β chain in a VαCαCκ/VβCβCκ heterodimer. Consistent with these results, Kearse and colleagues (39) have found that TCR β chains are markedly more stable than α chains in immature $CD4^+CD8^+$ thymocytes. This may have important implications concerning the structure of the $\alpha\beta$ heterodimer and signal transduction, possibly via an allosteric mechanism, which we discuss in more detail below.

3. ANTIGEN RECOGNITION

3.1 *Interaction with MHC/Peptide*

Models for the interaction between TCR and MHC/peptide antigen have been proposed independently by several groups (9, 36, 37); the models are based on the three-dimensional structure of the MHC/peptide moiety and the expected structural similarity between the TCR and an immunoglobulin Fab. All of these models propose that CDR1 and CDR2 of both the α and β chains of the TCR interact preferentially with the helices of the MHC molecule and that contacts to the bound peptide are made essentially by CDR3 alone. This appears to be borne

out by a number of mutagenesis studies of TCR/MHC/peptide interactions (10–15). The limited polymorphism of MHC molecules relative to the large diversity of antigenic peptides recognized by TCRs would indeed be consistent with this model in that a far greater diversity is present in CDR3 sequences by comparison with CDR1 and CDR2. Such a partition of function between the CDR as proposed by these models may restrain the orientation of the TCR with respect to the MHC molecule. Nonetheless, the proximity of some residues of CDR1 and CDR2 to CDR3 observed in the Vα (7) and Vβ (6) crystal structures would suggest that this partitioning of contact roles is probably not strictly adhered to. The CDR3 of both the α and β chains are less variable in length than those of the TCR γ and δ chains and of the immunoglobulin heavy chains. This would be compatible with proposed TCR/MHC/peptide models where the necessity of maintaining contacts with MHC as well as accommodating the volume of the peptide at the center of the antigen-binding site could place constraints on the size of the CDR3 (40). The observed shortening of β chain CDR3 in mouse by about one amino acid during the first week after birth would be consistent with some selective constraint operating on the naive immune system (41).

3.2 *Interaction with Superantigens*

While T cells respond to processed antigen in the form of peptide presented by the MHC class I and class II molecules, it is now well established that certain antigens do not require degradation in order to elicit stimulation. These molecules, known as superantigens (see 42, 43 for review), can appear either in soluble form, as with bacterial toxins, or as cell-bound products derived from certain retroviruses such as murine mammary tumor virus. They bind mainly to nonpolymorphic regions located on the Vβ domain rather than the CDRs. Thus, stimulation by superantigen leads to response in a significant portion of the T cell population (as much as 25–30%) corresponding to those carrying receptors coded by the superantigen-specific Vβ gene (or genes). Although MHC class II appears to be essential for stimulation by superantigens, mutation studies on the presenting molecule have suggested that interaction between the two species, at least in the case of staphylcoccal enterotoxin B (SEB) (44) and staphylococcal enterotoxin C (SEC) (45), does not involve the peptide-binding groove. This has indeed been confirmed by the recent crystal structure analysis of the binary complex HLA-DR1/SEB (46), which showed that the bacterial superantigen binds to the MHC α1 domain outside of the peptide-binding groove. Binding studies of the TCR/SEB/DR1 ternary complex confirm that that the association is not affected by the presence of different DR1-bound peptides (47). This mode of binding was thought to be more general since, by contrast with presentation of peptide antigens, presentation of superantigens did not seem MHC-restricted. More recently, however, the structure of the complex formed between

HLA-DR1 and toxic shock syndrome toxin-1 (48) shows that this superantigen covers approximately one half of the bound peptide, contacting some polymorphic residues on the $\alpha 1$ domain as well as on the $\beta 1$ domain.

Several studies have implicated certain TCR residues located on the putative solvent-exposed β sheet of the Vβ domain to be involved in superantigen binding, especially those on the β-hairpin formed by strands D and E of the third framework region (45, 49–53). In particular, an investigation into the reactivity of murine Vβ8.2 toward the self-superantigen Mls-1^{a} showed that stimulation could be significantly affected by mutations introduced at positions 19, 20, 24, 26, 73, and 74 of the TCR variable domain (49, 50). (These residues are numbered 17, 18, 22, 24, 70, and 71, respectively, by the authors.) Residues 19, 20, and 24 form part of the first framework region, and 26 belongs to CDR1, while 73 and 74 are located in the third framework region (18). These are on the surface of Vβ of the 14.3.d receptor (6) (the side chain of residue 19, however, is buried) and in principle should be capable of forming direct atomic contacts with the superantigen (Figure 5). Although Vβ8.2 of the wild-type strain (Vβ8.2a) is not stimulated by Mls-1^{a}, Vβ8.2b and Vβ8.2c from inbred strains do interact with the endogenous superantigen. Pullen and colleagues (49, 50) have found evidence that Asn24 and Asn74 of Vβ8.2a are probably glycosylated, and they proposed accordingly that carbohydrate could shield the Mls-1^{a}-binding site on Vβ8.2a, since mutation of these residues to the amino acids found in Vβ8.2b and Vβ8.2c (which do not carry the consensus N-glycosylation sequence) yields a receptor capable of interacting strongly with the superantigen.

Published models of TCR/SAg interactions (46, 49–53) have all taken the immunoglobulin V_H domain to base structural interpretations, but as discussed in a previous section, the HV4 region of Vβ8.2 is structurally closer to V_L than to V_H. The region of Vβ8.2 involved in superantigen binding should be similar in Vβ6, Vβ7, Vβ8.1, and Vβ8.3, with a possibility of Vβ9 as well, since all of these are expected to have the same canonical structure for CDR1 and CDR2, and they have a similar sequence in HV4, where Arg69, in particular, should play a critical role (see Table 1 and Figure 1*a*). This conclusion would be consistent with the negative selection of Vβ6, Vβ7, Vβ8.1, and Vβ9 by Mls-1a and of Vβ7 and Vβ8 by the staphylococcal enterotoxins *B, C3, D,* and *M. arthriditus* mitogen (55).

Superantigen binding was initially considered insensitive to the choice MHC because a given superantigen could be presented to the same TCR by different class II isotypes and alleles (56). Furthermore, CD8^{+} T cells expressing MHC class I–restricted TCRs recognize MHC class II/SAg complexes (57). Thus, TCR/MHC interactions were thought to be nonexistent, or at least

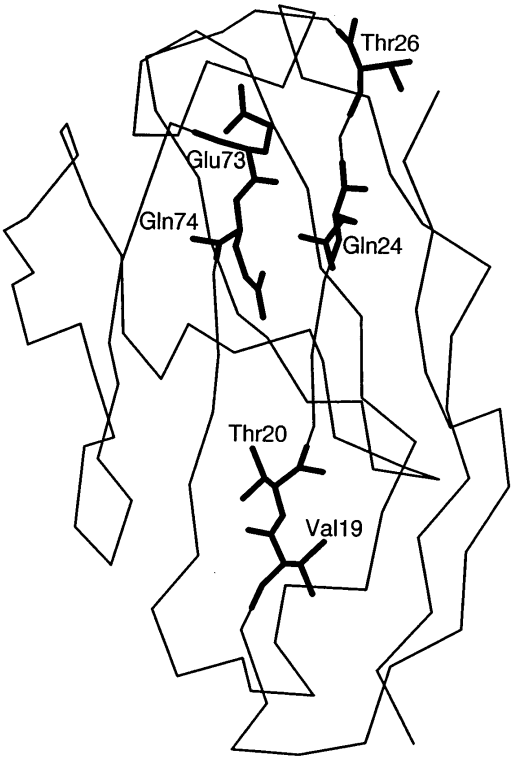

Figure 5 Residues on the Vβ8.2 domain that affect binding of Mls-1^{a} (49, 50).

unimportant, compared to TCR/SAg interactions in the ternary complex. Subsequent investigations of more weakly interacting TCRs have now provided strong evidence that TCR/MHC contacts probably modulate the fine specificities of superantigen binding as a function of MHC isotype and allele. For example, I-E molecules are more efficient than I-A molecules at presenting SEB to Vβ8^{+} T cells, to the extent that I-A^{q} molecules are incompetent (58, 59). Similarly, Woodland and colleagues (60) found that whereas Vβ8.2^{+} hybridomas varied significantly in their recognition of Mls-1 when presented by different MHC H-2 haplotypes, Vβ8.1^{+} hybridomas experienced no apparent modulation of response. Vβ8.1 and Vβ8.2 have the same sequence except in the CDRs and in five framework positions (10, 24, 83, 84 and 88). Furthermore, their CDR1 and CDR2 should have the same canonical form (identical CDR

lengths with key residues Gln25, Ser49, and Arg69). The different behavior of Vβ8.1$^+$ and Vβ8.2$^+$ T cells to superantigen response therefore suggests that direct contacts occur between the CDRs of the Vβ domain and the MHC molecule. Woodland and coworkers (60) also found supporting evidence that TCR/MHC interactions differ between superantigen and peptide antigen presentation since the preference for haplotype in Mls-1 fine specificity did not reflect the preference expected from positive selection in the thymus of the parental T cell that gave rise to the hybridomas.

Other studies have shown that residues from CDR1 of the Vβ domain affect the formation of the ternary TCR/SAg/MHC complex. In Vβ10 response to staphylococcal enterotoxin C2 and exfoliative toxin, Bellio and coworkers (54) examined the effect of mutation of CDR1 residues of Vβ10 between positions 26 and 31. Mutation at each position was able to suppress binding of at least one of the superantigens except for residue 28, which surprisingly provoked an increased response to both toxins. The CDR1 sequence of Vβ10 would suggest that its conformation is the same as Vβ8.2. This study could not differentiate TCR/SAg contacts from potential TCR/MHC contacts, but in the structure of Vβ8.2, the side chains of residues 28 and 30 appear to be most suitably placed for contact with the MHC/SAg moiety of the ternary complex.

Several studies have revealed an influence of the Vα domain upon the fine specificity of superantigen recognition (60–66). For example, by using mutants of the superantigen-presenting molecule, I-Eκ, contacts between Vα and the β chain of the MHC molecule could be inferred from the pattern of SEB stimulation of Vβ8.1$^+$ and Vβ8.2$^+$ hybridomas (66). Moreover, based on the crystal structure of the DR1/SEB complex (46), a model of the ternary complex TCR/SEB/DR1 was proposed in which mutagenesis data were used to deduce the TCR/SEB interface. This model indeed suggests that Vα/MHC contacts are a distinct possibility, and further, that the TCR/MHC contacts would have to be different in superantigen interaction since SEB covers β-chain residues of DR1, which are thought to be involved in conventional peptide antigen recognition.

3.3 *Superantigen Binding by the 14.3.d β Chain*

To determine whether soluble, unglycosylated 14.3.d β chain (Vβ8.2Jβ2.1Cβ1) retains SAg-binding activity, its affinity for various bacterial SAgs was measured in the absence of MHC class II molecules (67). Affinities were determined using two independent techniques: equilibrium sedimentation and surface plasmon resonance detection. Specific binding was demonstrated to SEB, SEC, and streptococcal pyrogenic exotoxin A (SPEA), consistent with the known stimulatory effects of these SAgs on Vβ8.2-bearing T cells (55, 68). Dissociation constants (K_D) ranged from 0.9 μM for SEC3 to 140 μM for SEB (see below). By contrast, SEA, which does not stimulate cells expressing Vβ8.2 (55), did

not bind the recombinant β chain with measurable affinity (67). Similar results were obtained for soluble, glycosylated 14.3.d $\alpha\beta$ heterodimer and for glycosylated β chain alone, indicating that α chain and carbohydrate do not contribute appreciably to SAg recognition in this particular case. From these studies, it may be concluded that the crystal structure of the 14.3.d β chain represents a biologically active conformation of this molecule, at least with respect to those regions that mediate binding to SAgs.

The much weaker binding of the 14.3.d β chain, and of the corresponding $\alpha\beta$ heterodimer, to SEB ($K_D = 140\ \mu$M) than to SEC1, SEC2, SEC3, or SPEA ($K_D = 2.5$, 2.3, 0.9, and 2.1 μM, respectively) was surprising, since SEB was found to actually be 3- to 10-fold more potent, on a molar basis, than the other toxins in stimulation assays using the 14.3.d hybridoma (67). These results may be interpreted in terms of the ability of SEC to activate T cells independently of MHC, in contrast to SEB (69). As discussed above, it is generally believed that SAgs activate T cells by simultaneously binding class II molecules on antigen-presenting cells and the Vβ element on T cells (43, 55, 70). Recently, however, through the use of class II knockout mice, SEC and SEE (but not SEB) were able to activate T cells in the absence of MHC (69). The responses resembled conventional class II–associated ones in that T cells expressing the same Vβ elements were selectively expanded: Vβ8-positive cells in the case of SEC and Vβ11-positive cells in the case of SEE. We attribute the ability of SEC to act in an MHC-independent fashion to its much higher affinity than SEB for Vβ8.2. That is, SAgs that bind TCRs with micromolar affinities may not absolutely require the participation of MHC class II molecules to stabilize their interactions with the β chain. However, it should be noted that, even for MHC-independent activation, APC from MHC-deficient mice were required for stimulation (69). This suggests that other surface molecules may substitute for MHC in binding certain SEs, thereby helping to stabilize TCR-SE interactions.

Binding of the 14.3.d β chain to all SAgs tested was characterized by very fast association rates ($> 1 \times 10^5\ M^{-1}s^{-1}$) and extremely fast dissociation rates ($> 0.1\ s^{-1}$) (67), the latter accounting for the rather weak affinities observed (relative to those of most antigen-antibody reactions). Fast dissociation kinetics have also been reported for the binding of the cell adhesion molecule CD2 to CD48 or CD58 (71, 72) and for the binding of TCRs to peptide/MHC complexes (73, 74). In the case of adhesion molecules, rapid dissociation rates may serve to facilitate de-adhesion, a requirement for cell motility (75). In the case of TCRs, however, fast dissociation rates would not appear to be compatible with a stable interaction with ligand believed to be necessary for signal transduction through the TCR/CD3 complex (74, 76). This implies that T cell activation by SAgs requires the participation of other surface molecules such as MHC in

order to stabilize the transient TCR-SAg interaction (58, 77–80). Multivalent binding arising from receptor oligomerization (see below) could also increase the lifetime of TCR-SAg complexes. Another possibility is that fast dissociation rates may actually be critical for T cell triggering by SAgs, as suggested by a recent study in which a single peptide/MHC complex was found to serially engage and downregulate nearly 200 TCRs (81). Superantigens bound to the surface of APC may display similar behavior.

In contrast to the mouse Vβ8.2 TCR described above, a soluble human Vβ3.1 TCR binds SEB with association and dissociation rates that were only moderately fast: 1.3×10^4 M^{-1} s^{-1} and 1.1×10^{-2} s^{-1}, respectively (47). On the other hand, SEB was found to bind HLA-DR1 with rapid kinetics very similar to those of the Vβ8.2-SAg interaction. This suggests that binding of human Vβ3.1 to SEB helps stabilize the transient SEB-DR1 interaction and, as a result, the TCR-SEB-DR1 complex (47). If so, one might expect the transient binding of murine Vβ8.2 to SEB to be stabilized by a more kinetically favorable interaction of SEB with I-E or I-A molecules, that is, one with a slower dissociation rate. These results suggest that the same SAg may activate different T cells by different mechanisms, depending on the particular Vβ element they express and on the specific MHC background of the host.

As mentioned above, carbohydrate does contribute to enterotoxin binding by the 14.3.d β chain (67). This is rather surprising, since the potential N-linked glycosylation sites at Vβ residues 24 and 74, which were mutated to glutamine, are at or near the putative SAg binding site in the crystal structure of the β chain (6). Indeed, Asn74 is part of HV4, which has been directly implicated in SAg recognition (70). At least one of these residues appears to be glycosylated (50), suggesting that they may actually lie at the periphery of the binding site for bacterial SAgs. It is worth noting in this respect that mutation of Asn24 to glutamate confers reactivity to Mls-1 (49, 50), suggesting that viral and bacterial SAgs bind to somewhat different regions of Vβ.

Pairwise comparisons between mouse Vβ8.2 and other Vβ regions based on the crystal structure of the former have been carried out in order to investigate the structural basis for reactivity with different bacterial SAgs (67). As might be expected, Vβ8.2 shares a larger number of conserved residues on its putative SAg-binding face with Vβs with similar SE reactivity profiles (e.g. Vβ3, Vβ7, Vβ11) than with Vβs with very different ones (e.g. Vβ1). Surprisingly, however, each pairwise comparison revealed a different set of conserved residues, even between Vβ8.2 and Vβs, which react with a similar spectrum of SAgs; furthermore, these residues are not particularly concentrated in HV4. Thus, certain SAgs (e.g. SEC3, which reacts with Vβ3, Vβ7, and Vβ8.2) are able to recognize quite different molecular surfaces on different Vβ domains. This

could occur if individual SAgs possessed several distinct Vβ-binding sites. Several precedents, however, suggest that it is not necessary to postulate multiple binding sites to account for the recognition of topographically distinct surfaces by a single molecular species (82–84).

4. TCR DIMERIZATION AND T CELL ACTIVATION

Ligand-induced dimerization (or oligomerization) appears to be a general feature of the activation of cell-surface receptors (85, 86). Examples include cytokine receptors, protein-tyrosine kinase receptors, serine/threonine kinase receptors, and receptors of the tumor necrosis factor family. Certain ligands activate their receptors in soluble form (e.g. growth hormone, epidermal growth factor), while others require membrane attachment, as in the case of ligands for EPH-related tyrosine kinase receptors (87). There is considerable evidence that antigen receptors, both membrane immunoglobulin and TCRs, must also undergo multimerization for signal transduction to occur (88, 89). In particular, it has long been known that T cells can be triggered by divalent antibodies specific for TCR or CD3 determinants, but not by their monovalent Fab fragments (90, 91, 92). More recent evidence derives from experiments involving chimeric molecules consisting of the extracellular and transmembrane regions of non-TCR cell-surface proteins linked to the cytoplasmic regions of CD3 ϵ (93) or ζ chains (94, 95). Upon cross-linking with specific monoclonal antibodies, the chimeras were capable of transducing signals similar to those generated by the intact TCR, suggesting that juxtaposition of CD3 subunits is required to promote cross-phosphorylation of tyrosine kinases associated with the TCR/CD3 complex, in common with other receptor systems (85, 86).

These studies, along with the finding that HLA class II molecules crystallize as parallel dimers (96), led to a model for T cell activation in which class II dimers facilitate TCR dimerization, resulting in signal transduction (96, 97). A limitation of this model, however, is that there is no known mechanism for generating class II dimers loaded with identical peptides, making it difficult to explain how two TCRs can be brought into juxtaposition by as few as 100 specific peptide/MHC complexes among the roughly 100,000 irrelevant complexes found on the surface of a typical APC (98–100). Since an individual T cell generally expresses only a single $\alpha\beta$ heterodimer, an obvious solution to this problem would be if the TCR itself had a tendency to dimerize (96, 97).

The recently determined crystal structure of the Vα domain of a TCR specific for the N-terminal nonapeptide of myelin basic protein in association with I-A^u provides evidence for such dimers of dimers (7). The Vα domain exists as a homodimer in solution and in the crystals (101) analogous to the known structures of immunoglobulin light chain dimers (102) and the T cell co-receptor

CD8 (103). However, a switch in a polypeptide strand from one β sheet to the other distinguishes the Vα secondary structure from that of antibody V domains and from the TCR Vβ domain (7). This switch results in the removal of a surface protrusion and enables a pair of Vα homodimers to pack together to form a tetramer such that the two homodimers are parallel to each other and all CDR loops face in one direction. A model of an $(\alpha\beta)_2$ TCR tetramer constructed on the basis of the observed mode of Vα dimer-dimer association and the β chain structure (6) shows structural complementarity to the HLA-DR1 $(\alpha\beta)_2$ tetramer (96).

Assuming that the parallel dimers observed in Vα crystals represent a physiologically relevant interaction, a mechanism for T cell activation may be envisaged whereby α chain–mediated TCR dimerization drives formation of a homogeneous peptide/MHC class II dimer; binding of the class II dimer in turn stabilizes the weak TCR dimer sufficiently to trigger the receptor. In the absence of cognate peptide/MHC complexes, however, the putative TCR dimers exist only transiently on the cell surface, thus preventing T cells from triggering spontaneously. This cooperative mechanism, whereby weak TCR dimers interact with weak class II dimers, resulting in the formation of stable TCR_2-MHC_2 signaling complexes, differs from that of most other receptor systems in which the ligand either exists as a stable, preformed dimer (platelet-derived growth factor, stem cell factor), or itself possesses two independent receptor-binding sites (growth hormone) (83, 86, 104).

The role of TCR dimerization in T cell activation has been recently questioned (81), because T cell clones expressing two different TCRs show that only the TCR specifically triggered by antigen (TCR_A) is downregulated, while the irrelevant TCR (TCR_B) is unaffected. One interpretation of these data is that TCRs do not self-associate and that they engage peptide/MHC in a monovalent fashion (81). An alternative explanation is that only TCRs whose dimerization is stabilized by specific interactions with cognate peptide/MHC complexes undergo activation. In this way, only TCR_A-TCR_A homodimers can go on to form productive signaling complexes, whereas TCR_A-TCR_B heterodimers cannot, at least in the absence of peptide/MHC complexes specific for TCR_B. Homotypic association of TCRs could also contribute to the results of Valitutti and colleagues (81), although we do not consider this to be the primary mechanism. An additional finding was that a single peptide/MHC complex can sequentially engage and trigger as many as 200 TCRs (81), consistent with the low affinities and rapid dissociation rates that have been measured for TCR binding to peptide/MHC (73, 74). While bivalent binding arising from receptor dimerization would effectively decrease the off-rates (and increase the half-time) of TCR-peptide/MHC interactions, we do not believe this

is incompatible with a small number of peptide/MHC complexes successively ligating, triggering, and dissociating from a large number of TCRs. Indeed, recent crystallographic evidence indicates that cell-adhesion molecules, which also appear to rely on fast dissociation rates to facilitate de-adhesion (71, 72, 75), organize into cooperative, multimeric superstructures to strengthen intercellular bonds (105). Thus, the serial engagement model for T cell triggering (81) is compatible with one requiring TCR dimerization for each activation event.

The possibility that T cell signaling also involves conformational changes in the TCR has been discussed by Karjalainen (106) and Janeway (107). While bivalent antibodies are required to trigger T cells, the Fab fragment of one particular anti-TCR antibody induces the physical association of the TCR with CD4 and CD45 on the T cell surface, which is believed to be a critical step in T cell activation (108). One interpretation of these results is that the monovalent Fab fragment, though unable actually to activate the T cell, induces a conformational change in the TCR similar to that associated with the binding of MHC/peptide. In the crystal structure of the β chain, the close association observed between the Vβ and Cβ domains could facilitate the transmission of any structural changes that may occur in the TCR upon antigen binding to associated CD3 molecules in the TCR/CD3 complex. Direct structural studies of the TCR in free and liganded forms will be required to resolve these issues unambiguously.

5. CONCLUDING REMARKS

The crystallographic analyses of TCR fragments discussed in this review have given preliminary insights into the three-dimensional structure of the receptor. They have suggested the existence of certain patterns to the conformation for the CDR, which await testing by further crystallographic studies on the $\alpha\beta$ (and $\gamma\delta$) heterodimers and their fragments. These patterns, which differ in some aspects from those found in immunoglobulins, appear consistent with certain proposals for TCR/MHC/peptide interactions. While we may hope for structural analyses of the TCR/antigen complex in the future, the present results provide a base for designing further experiments to probe the nature of TCR/antigen interaction by other means. In addition, these three-dimensional structures have offered some indications of the mechanism that might operate in T cell activation; these, likewise, are now open to experimental testing. Given the complexity of the membrane-bound TCR/CD3 system, the contribution of structural studies may remain reductionist in its approach for some time, perhaps mainly providing structures of individual extracellular and cytoplasmic domains of the TCR/CD3 complex in isolation from its other components, its

antigen and the coreceptors. Nonetheless, such studies will offer an important addition to our understanding of the rather complex recognition and signaling system.

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IMMUNE REGULATION BY CD40 AND ITS LIGAND GP39

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ABSTRACT

Over the past three years, CD40 and its ligand (gp39, CD40L, TBAM) have been shown to be essential for humoral immune responses to thymus-dependent antigens. However, as the tissue distribution widens for those cells that express CD40 and gp39, we can now show that this ligand-receptor pair also plays an important role in the selection of self-reactive T cells in the thymus (central tolerance) and the regulation of tolerance in mature T cells (peripheral tolerance). Advances in our understanding of the molecular basis for CD40 biology is based in two areas of research. First, a major breakthrough in our understanding of how CD40 transduces biological events centers on the identification of a novel protein that binds to the cytoplasmic tail of CD40 and may act as a signal transducing molecule. Secondly, advances in molecular modeling and mutagenesis of this ligand-receptor pair have helped to identify the critical receptor/ligand contacts in the gp39/CD40 complex. Advances in each of these areas are discussed.

1. INTRODUCTION

A number of lines of investigation have led to the conclusion that CD40 and its ligand gp39 are critical for the development of humoral immunity. Loss of gp39 function results in a near total loss of thymus-dependent primary and secondary humoral immune responses and the development of B cell memory. Recent

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studies show that functional CD40 expression is more widely distributed than previously thought, and as such, blockade of gp39 function affects many arms of the immune response originally thought not to involve this ligand-receptor pair. The induction of peripheral tolerance and the selection of self-reactive T cells are both influenced by blockade of gp39-CD40 interactions. The role of CD40 and its ligand are addressed with an emphasis on in vivo studies since, recently, Banchereau and coworkers (1) have published an excellent, extensive review of the role of CD40 in the regulation of human B cell growth and differentiation in vitro.

2. CD40 STRUCTURE, DISTRIBUTION, AND SIGNALING

CD40 is a member of the tumor necrosis factor receptor (TNF-R) family of receptors that includes TNF-R1, TNF-R2, p75 nerve growth factor receptor (NGFR), CD30, CD27, Fas, and others. CD40 is a 50-kDa integral membrane protein whose tissue distribution was originally thought restricted to B cells, dendritic cells, and basal epithelial cells (2), but more recently it has been shown to be functionally expressed on macrophages (3), Langerhan cells (4), endothelial cells (5, 6), and thymic epithelial cells (7). This broadening of CD40 distribution increases the range of tissues that gp39-bearing effector T cells can influence.

2.1 *CD40 Signaling*

The predicted cytoplasmic domain of CD40 is 65 amino acids, and there are no recognizable homologies to kinases or other sequences that would link the molecule to a canonical signal transduction cascade. However, mutagenesis studies (8) indicate that the Thr234 residue in the cytoplasmic domain is critical for signal transduction. Only a limited number of studies have taken traditional approaches to identify signal transduction cascades involved in CD40 signaling. In murine systems, it has been reported (9, 10) that plasma membrane preparations from T cells expressing gp39 induced the protein tyrosine phosphorylation (pTyr) of a heterogeneous group of proteins in resting B cells. Furthermore, it appeared that activation of protein kinase C (PKC) did not occur upon CD40 engagement. Following recognition that gp39 is a critical component in T cell–dependent B cell activation, interest turned to using anti-CD40 mAbs (instead of complex plasma membrane preparations containing gp39) to trigger biochemical events.

A number of studies have attempted to use anti-CD40 to trigger pTyr in resting B cells. In one study (11), while activated B cells triggered pTyr events, under identical conditions, no pTyr events were observed in anti-CD40-triggered resting B cells. Another study using dense, tonsillar B cells demonstrated the

pTyr of a 28-kDa band upon CD40 cross-linking (12). It is not clear at this time why resting B cells do not effectively trigger pTyr cascades in response to CD40 ligation, but do so in response to gp39-bearing plasma membranes. This may suggest that other membrane proteins are critical to activation of resting B cells via CD40. In this context, Kehry and coworkers (13) have shown that an activity other than gp39 is important for maximal activation of resting B cells by gp39-bearing membranes. Along these same lines, to achieve the growth of limiting numbers of resting, human B cells in response to anti-CD40, fibroblasts must be included in the culture (1). It is likely that these fibroblasts are fulfilling a contact-dependent function in supporting B cell growth and differentiation.

Greater success in mapping out the CD40 signal transduction cascade has been seen in studies using transformed B cell lines and activated, normal B cells. Anti-CD40 triggering of a panel of B cell lines (representing pro-B-, pre-pre-B-, pre-B-cells), or activated mature B cells with an agonistic anti-CD40 mAb, results in enhanced pTyr of a number of distinct phosphoproteins and induces a rapid increase in the production of inositol 1,4,5-trisphosphate (IP_3). These same investigators also showed that CD40 engagement activates a number of serine/threonine-specific protein kinases. Interestingly, inhibitors of tyrosine-specific protein kinases abrogated the CD40-induced pTyr, CD40-induced stimulation of IP_3 turnover and CD40-induced, serine/threonine-specific protein kinase activity. More recently, CD40 cross-linking was shown to induce a rapid and sustained increase in the phosphorylation and activity of the src kinase lyn in Daudi B cells (14). The activation of lyn appeared selective because the activities of fyn, fgr, and lck kinases were minimally changed following CD40 engagement. CD40 engagement also resulted in phosphorylation of phospholipase Cγ2 (PLCγ2) and phosphatidylinositol (PI)-3-kinase. Similar approaches have been taken to elucidate the CD40 signal cascade in the human B cell lines by Raji & Ramos (12). These studies presented different patterns of activity following CD40 triggering. These investigators reported that there was a marked and rapid tyrosine phosphorylation of an unidentified 28-kDa protein, yet a marked, early (30 sec) dephosphorylation of lyn, fyn, and syk. These three studies all document heterogeneous patterns of pTyr in response to CD40 cross-linking in B cell lines, and those that examined resting B cells, with one exception (14), could not document any increased pTyr. Therefore, little success has been reported in using anti-CD40 to trigger a cascade of biochemical events in ex vivo B cells, except in germinal center (GC) B cells (15) (see below).

At the time that studies were undertaken in EBV-infected, human B cell lines, it was not known that EBV transforms human B cells by inducing the overexpression of the putative signaling chain of CD40 (see below) and organizing it into clusters in the lymphoblast plasma membrane (16). It is believed that

the organization of the signaling chain into these clusters significantly contributes to transformation. Therefore, one would imagine that measuring CD40-transduced events in the context of a chronically engaged CD40 signaling cascade would be difficult. Furthermore, although CD40 engagement on ex vivo resting B cells and dense, tonsillar B cells exerts profound biological effects (growth and anti-apoptosis, respectively), it is not clear what biological signals are transduced by CD40 in the B cell lines used in the studies cited above. Finally, it is curious that in resting, naive, μ, δ^+ B cells, where the biological consequences of CD40 engagement are profound, few early signaling events have been directly identified using recombinant, soluble gp39 or anti-CD40. Therefore, whether CD40-induced events observed in B cell lines are responsible for the induction of resting B cell activation by CD40 engagement is yet to be determined.

Although CD40-induced signaling cascades in ex vivo B cells have been difficult to resolve, some early events have been documented. Rothstein and coworkers (17) have demonstrated that T cells expressing gp39 could induce the activation of the *trans*-acting transcription factor nuclear factor κB (NFκB) in resting B cells. Interestingly, depletion of protein kinase C (PKC) in B cells diminished anti-Ig-induced NFκb activation but not CD40-induced activation. With regard to other prominent transcription factors, both forms of stimulation led to expression of JunB and JunD mRNA and protein; however, induction of JunB mediated by anti-Ig Ab was protein kinase C (PKC)-dependent, whereas induction mediated by CD40 ligand was independent (18). Both anti-Ig and CD40 engagement have been shown to induce c-Fos; however, expression of FosB was only induced by anti-Ig. The authors concluded that one can observe receptor-specific differences as well as similarities in the induction of transcription factors by membrane Ig and CD40.

In addition to the role of CD40 in expansion and differentiation of resting B cells, CD40 cross-linking is also an extremely efficient means to prevent apoptosis in GC B cells (19) as well as in immature B cells (20). Cross-linking CD40 on GC B cells resulted in enhanced pTyr on heterogeneous substrates. In this case, the pattern of pTyr was similar to that observed upon cross-linking mIg (15). Although anti-Ig induced an increase in inositol 1,4,5-trisphosphate (IP_3) and cytosolic free calcium in both GC B cells and resting B lymphocytes, anti-CD40 failed to do so. Through the use of pTyr kinase inhibitors, the investigators were able to show that pTyr is mandatory for CD40-mediated rescue of GC B cells from apoptosis. Therefore, it is reasonable to conclude that CD40 is linked via some mechanism to an early activation of a protein tyrosine kinase (PTK) cascade, at least in GC B cells, and this cascade may be involved in the anti-apoptotic effects of anti-CD40.

2.2 *CD40 Binding Protein*

A substantial advance in our understanding of CD40 signaling emerged in the past year with the identification of a protein that binds to the cytoplasmic domain of CD40 (21–23). Using the yeast two-hybrid system, Dixit and coworkers (21) first identified a novel RING finger protein that interacts with the cytoplasmic domain of CD40. The CD40 binding protein (CD40bp) is homologous to two cytoplasmic proteins that were recently shown to bind to the cytoplasmic domain of the TNF-R (TRAF-1 and TRAF-2). CD40bp has three structural domains, a c-terminal TRAF-homology domain, a central coiled-coil and a n-terminal cysteine-histidine–rich RING finger, DNA-binding motif. The binding of CD40bp to the cytoplasmic domain of CD40 was lost upon mutation of the functionally significant Thr234 residue within CD40. Subsequently, the c-terminal TRAF domain was shown to be necessary and sufficient to bind to the CD40 cytoplasmic tail. In addition, overexpression of the truncated TRAF domain exerted a dominant negative effect on the CD40-inducible upregulation of CD23, a CD40-regulated membrane protein (23). Interactions of CD40bp are specific to CD40 in that CD40bp did not bind to TNF-R1, Fas, or TNF-R2 (21–23). The discovery of the TRAF family of signal transducing molecules represents a substantial stride in our understanding of TNFR family signaling, and there is good indication to believe that the downstream signaling elements will be identified shortly.

3. GP39 STRUCTURE AND REGULATION OF EXPRESSION

3.1 *Molecular Modeling and Mutagenesis Studies on gp39 and CD40*

Crystallographic structures of gp39, CD40, and a gp39/CD40 complex are yet to be determined; however, the X-ray structures of the homologous molecules TNF-α, TNF-β (24–26), and the TNF-β/TNFR complex have been reported (27). These structures have served as templates to prepare three-dimensional models of the extracellular regions of gp39 (28–30), CD40, and the gp39/CD40 complex (31). These models predict that gp39 forms a trimer and that CD40 binds to the gp39 trimer at the interface between two monomers. Analogous to the TNF-β/TNFR system, these models predict that each gp39 trimer is capable of binding to three CD40 molecules (31). In the TNF/TNFR system, ligand binding has been proposed to result in receptor oligomerization and signal transduction (27, 32, 33). The results of the gp39/CD40 molecular modeling studies suggest that a similar mechanism might be in part responsible for gp39-dependent CD40 signaling.

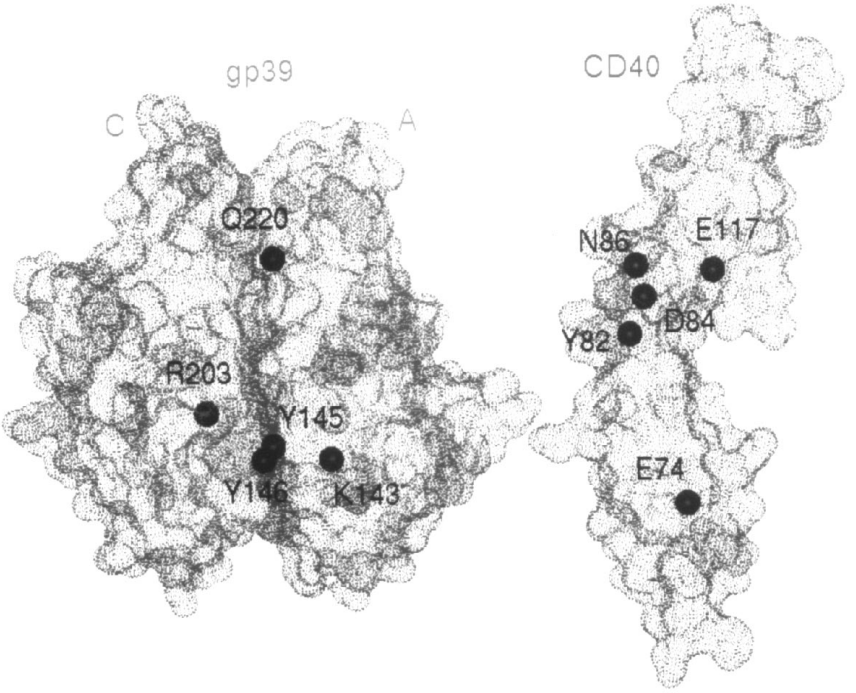

Figure 1 Residues critical for the gp39-CD40 interaction. The α-carbon positions of gp39 and CD40 residues identified by targeted mutagenesis as significantly contributing to the ligand-receptor interaction are mapped on the gp39 and CD40 molecular models (shown in solvent-accessible surface representations and described in the text). The analysis suggests that residues of two adjacent monomers of a gp39 trimer (here A and C) form a CD40 binding site. Gp39 and CD40 are shown facing the predicted contact sites.

The gp39 and CD40 models were used to select surface residues in gp39 and CD40 to be targeted for site-directed mutagenesis. Some of these residues are found in positions corresponding to residues in TNF and TNFR that participate in the formation of the TNF/TNFR complex. Binding studies with the mutant proteins resulted in the identification of five gp39 residues (K143, Y145, Y146, R203, and Q220) and five CD40 residues (E74, Y82, D84, N86, and E117) whose replacement with Ala substantially affected gp39/CD40 interactions (Figure 1) (29, 31). Additional mutagenesis and binding studies, in which gp39 residue Y145 and CD40 residue Y82 were replaced with Phe instead of Ala, showed that the gp39 mutant Y145F bound CD40 at levels comparable to wild-type gp39, whereas the CD40 mutant Y82F did not bind gp39 (31). These

studies illustrate that the absence of a hydroxyl group on CD40 residue Y82 is sufficient to disrupt the gp39/CD40 interaction. In all cases the structural integrity of the mutant proteins was monitored using a panel of anti-gp39 and anti-CD40 mAbs, recognizing different conformational epitopes prior to their use in the binding studies.

Further structural information has been obtained by studies of the defects in gp39 that lead to HIM syndrome in humans (30, 34–36). In many instances, these defects are the result of point mutations in the extracellular domain of gp39. A three-dimensional model–based analysis of the location of naturally occurring point mutations (as reported in the literature before mid 1994) suggests that one gp39 mutation, E129G, affects a solvent-accessible residue (29). This mutation was identified in a patient whose gp39 was found to contain the double mutation S128R-E129G (30). To assess the individual contribution of these substitutions on the gp39/CD40 interaction, two point mutations were prepared—S128R and E129G (29). Binding studies with these two point mutants showed that both mutations significantly reduced the ability of gp39 to bind CD40. The replacement of E129 by Gly results in the loss of a negatively charged side chain, which may function as a CD40 contact residue but also increases the flexibility of the molecule in this region. To examine the individual contribution of these changes, an additional gp39 mutant, E129A, was prepared. The binding of this gp39 mutant to CD40 was identical to that of wild-type gp39. These results indicate that gp39 residue E129 is not directly involved in CD40 binding, but that the naturally occurring E129G mutant causes a structural perturbation in gp39 that affects CD40 binding.

In summary, the results of these modeling and mutagenesis studies suggest that clusters of residues in two adjacent gp39 monomers are involved in CD40 binding (Figure 1). The regions identified by these clusters of residues correspond to those found to be involved in the binding interaction in the homologous TNF-β/TNFR complex. The finding that the residues involved in forming critical receptor/ligand contacts in the gp39/CD40 and TNF-β/TNFR complexes are not conserved provides a molecular rationale for the receptor/ligand binding specificity observed in this family of closely related receptor/ligand pairs.

3.2 *Regulation of gp39 Expression*

Pharmacologic activation of $CD4^+$ T cells or engagement of the TCR with mAbs induces a transient expression of gp39 (37, 38, 39). In addition to $CD4^+$ T cells, gp39 can reportedly be inducibly expressed by some $CD8^+$ T cells (38) and some $CD8^+$ clones (40), as well as by γ, δ T cells (37, 41). In vivo, the administration of a TD antigen induces the expression of gp39 on predominately $CD4^+$ T cells, which are found juxtaposed to B cells producing

antibodies to the immunizing antigen (42), suggesting that gp39 is expressed during T-B interactions. With the use of T cell clones, gp39 has been shown to be expressed on activated Th0 and Th1, as well as on Th2 type cells (37, 43). The patterns of gp39 expression on activated memory T cells and on naive T cells appear similar (37) and are sensitive to cyclosporin (37, 44). In addition to the expression of gp39 on activated $CD4^+$ T cells, gp39 is also expressed on activated basophils (45) and eosinophils (46). In both of these cases, these activated cells could mediate the induction of IgE production by B cells in the presence of IL4. Therefore, as with CD40, the scope of cells capable of expressing the ligand has broadened since its discovery.

Early studies used polyclonal activators to induce gp39 and did not provide any insights into the contribution of adhesion or costimulatory molecules to the antigen-induced expression of the molecule. In experiments designed to better understand how antigen regulates the expression of gp39, Roy et al (47) studied the regulation of gp39 expression on pigeon cytochrome C (PCC) TCR transgenic (Tg) T cells in vitro (Figure 2). In the presence of APC, the addition of PCC induced gp39 expression on the Tg T cells, and this expression was inhibited by antibodies to class II, CD4, or LFA-1. Agents that blocked B7, including anti-B7.1, anti-B7.2, and CTLA 4-Ig, did not interfere with the antigen-induced upregulation of gp39. These results suggest that engagement

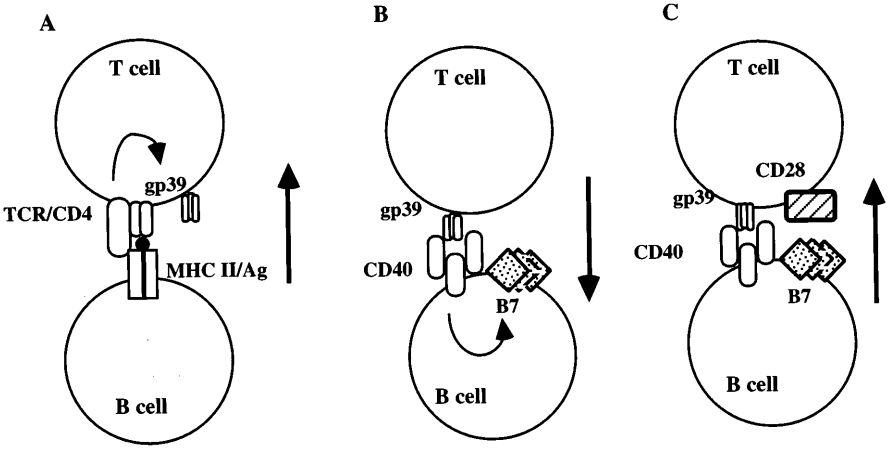

Figure 2 Engagement of the TCR by antigen induces gp39 expression. Interaction of the TCR with Ag-MHC complex on the APC, together with LFA-1, induces the expression of gp39 on the responding T cell (*A*). Expression of gp39 results in the engagement of CD40 on the cognate B cell causing the upregulation of B7.1 and B7.2 (*B*). As a consequence of increased expression of B7.1 and B7.2, the B cells reciprocally trigger the T cell via CD28 (*C*).

of the TCR by Ag-MHC complex induces the expression of gp39, and that costimulation by B7-CD28 pathway was not required for the expression of gp39 on naive T cells. These findings accord with the ability of anti-CD3 alone to effectively induce gp39 expression on ex vivo T cells and clones. In a contrasting study, de Boer et al showed that human T cells activated with mAb to the TCR/CD3 complex required a CD28-B7 signal for the upregulation of gp39 (48). Resolution of the discrepancies in these two reports is not apparent at this time; however, B7-CD28 interactions may contribute to gp39 expression under certain conditions of TCR occupancy.

The system employing PCC TCR Tg T cells and B cells (as the APC) also afforded the opportunity to study the impact of gp39 on the expression of costimulatory molecules by the cognate B cells. As a result of PCC-induced expression of gp39 expression on the PCC TCR Tg T cells, the cognate B cells were induced to express B7.1 and B7.2. This is consistent with a number of previous studies showing that gp39 could upregulate B7 expression on B cells (49, 50). Therefore, in addition to its central role in initiating the clonal expansion and differentiation of B cells, gp39 is also critical for B cells to express high levels of costimulatory molecules and for B cells to mature to "competent" APCs. Having matured, the B cells can now reciprocally trigger T cells via costimulatory molecules to maximize the signaling of T cell growth and lymphokine production (Figure 2).

4. THE ROLE OF CD40 IN THE REGULATION OF HUMORAL IMMUNITY

The genetic evidence that provided conclusive proof that gp39-CD40 interactions were essential for thymus-dependent (TD) humoral immune responses was derived from studies in humans with the immunodeficiency, hyper IgM syndrome (HIM) (30, 34–36). The genetic basis of this disease is that mutations in the gp39 gene cause alterations in the gp39 protein that render it incapable of binding to CD40 (see previous section). As a result, HIM helper T cells cannot trigger B cell activation and immunoglobulin production. This disease is characterized by reduced, if not absent, levels of serum IgA, IgG, and IgE. Furthermore, these patients have a complete absence of GC in their secondary lymphoid organs. In cases where a mutation in gp39 has been documented, the B cells from these patients are functional in vitro. However, a subset of HIM patients exists that express wild-type gp39 but whose B cells have defects in CD40 signaling (51).

Over the past three years, insights into the function of gp39 and CD40 in the regulation of humoral immunity have been provided by studies in gp39- (52, 53)

and CD40-deficient (54, 55) mice and in mice treated with anti-gp39 (42, 56, 57). For the most part, all of these systems agree that gp39 interactions are essential for secondary immune responses to TD antigens and in the formation of GC (Table 1). Studies using anti-gp39 (57) or a soluble form of CD40 (58) to inhibit the in vivo function of gp39 show that the generation of memory B cells is also dependent on this ligand-receptor pair. There is controversy as to whether gp39 is essential in GC development because the administration of CD40-Ig did not, but anti-gp39 did, block GC formation (57, 58). In addition, there is unanimous agreement on the lack of involvement of this ligand-receptor pair in the intensity and isotype distribution of humoral immune responses to most T-independent antigens. One aspect in which these systems diverge is the role of gp39-CD40 interactions in the regulation of primary immune responses (IgM) to TD antigens. Some, but not all, show that gp39-CD40 interactions are important in IgM responses. Therefore, this offers the possibility that molecules other than gp39 can serve as "helper" effector molecules in humoral immunity. In this regard, recent reports (59, 60) have indicated that helper T cells or clones from HIM patients can, in some instances, induce IgE production and IgM. Therefore, one could speculate that molecules other than gp39 may play a minor role in supporting some TD humoral immune responses.

Although it is evident both from genetically deficient mice and from mice treated with anti-gp39 that gp39-CD40 interactions are critical for the formation of germinal centers, recent evidence suggests that gp39 also is important in the maintenance of GC. Kelsoe and coworkers (61) have shown that even after GC have formed, anti-gp39 treatment can cause their precipitous loss. It has been well established that GC B cells acutely apoptose upon isolation and that CD40 ligation can "rescue" them from death (19). Therefore, the immediate assumption is that anti-gp39 treatment causes the loss of GC due to increased apoptosis. We have reproduced the findings of Kelsoe and coworkers; however, we cannot find any evidence that anti-gp39 treatment causes an increase in GC apoptosis (JD Laman, unpublished data). It is clear that gp39 is likely playing

Table 1 The role of gp39-CD40 interactions in humoral immunity

gp39-dependent
Primary immune responses to TD antigens
Secondary immune responses to TD antigens
Generation of memory B cells
Generation of GC
Maintenance of GC
gp39-independent
Titer and isotype distribution of antibody responses to TI antigens

an important role in GC maintenance, and the question emerges as to why treatment with anti-gp39 causes their profound and rapid loss. It is possible that loss of gp39 function in the GC causes B cells to traffic to the bone marrow and terminally differentiate into plasma cells. This hypothesis would be consistent with a recent in vitro study that shows that the withdrawal of gp39 results in memory B cells terminally to differentiate to plasma cells (62).

Since it is clear that gp39 plays an important role in germinal center homeostasis, it is important to determine which cells within the GC are capable of expressing gp39. Early studies in immune mice did not detect gp39 in the GC of immune mice (42); however, other studies in human tonsil have indicated its presence (63, 64). Most recently (64), in human tonsil, a subset of T cells was discovered to contain preformed gp39 that can be mobilized rapidly upon activation. The T cells containing preformed gp39 were found predominately in the outer zone of the GC and could be important cells for the maintenance of GC integrity. The basis for different results in murine and human systems may be species differences, the use of immune spleen from mice vs chronically stimulated or inflamed tissue from humans, or the lack of sensitivity of the anti-murine reagent.

5. THE ROLE OF GP39 IN SHAPING THE T CELL REPERTOIRE

The detection of CD40 within the thymus has led to the suggestion that interactions between CD40 and its ligand may be of functional importance in the selection of the T cell repertoire (21). CD40 is expressed in the thymus on a number of cell types including cortical and medullary epithelial cells, interdigitating cells, dendritic cells, macrophages, and B cells (7, 65). The fact that many of these cell types mediate selection of thymocytes (66) supports the hypothesis that gp39-CD40 interactions may play a role in thymic selection. However, until recently, no studies have directly addressed the functional role of this receptor-ligand pair in the process of thymic selection. A brief discussion of thymic selection and evidence for the potential role of CD40, gp39, and other costimulatory molecules is presented in this section.

5.1 *Thymic Selection*

The T cell repertoire is shaped in the thymus as a consequence of TCR gene rearrangement, selection and survival of thymocytes bearing TCRs that are able to bind self-MHC molecules (positive selection), and deletion of thymocytes bearing TCRs exhibiting self-reactivity (negative selection). It is now widely accepted that TCR recognition of MHC/peptide by mature peripheral T cells is insufficient for optimal T cell activation and that costimulatory molecules

provided by APCs are necessary to achieve complete T cell activation (reviewed in 67). Although it is clear that both positive and negative selection in the thymus occur as a result of MHC/peptide recognition by TCR, the requirement for costimulatory molecules in these processes is still in doubt. While the majority of studies suggest that TCR signals alone are sufficient to induce deletion of thymocytes, other costimulatory molecules have been implicated as participants in negative selection.

Because of the well-documented function of CD28/B7 interactions as costimulatory for activation of peripheral T cells, a number of studies have examined the role of CD28/B7 family members in thymocyte selection. CD28 is expressed on thymocytes in highest amounts at the CD4/CD8 double positive (DP) stage, a stage at which developing T cells are undergoing selective events (68). This developmental regulation of CD28 expression supports the notion that it may be involved in thymocyte selection. Two studies provide additional support that the CD28/B7 signaling pathway may be involved in negative selection. A causal role for B7 in negative selection is suggested by recent studies demonstrating that the expression of B7 within the thymus medullary epithelium was T dependent and correlated with epithelium-mediated deletion of $V\beta5^+$ thymocytes (69). More direct evidence comes from in vitro studies demonstrating that CD28 could provide an apoptotic signal to immature DP thymocytes (70). However, other studies have provided conflicting data. Page and coworkers demonstrated that an APC-derived costimulatory signal was required for deletion of DP thymocytes in vitro. The authors concluded, however, that this costimulatory signal was not provided by B7 (71). Studies examining CD28 and B7 knockout mice as well as those utilizing CTLA4 have failed to support a unique role for these molecules in thymocyte selection (72–74). Moreover, in vitro studies utilizing CTLA-4 or anti-CD28 antibodies have also led to the conclusion that these costimulatory molecules are not required for deletion of thymocytes in fetal thymic organ cultures (75, 76). Given the number of different model systems used in the studies presented above, each utilizing unique mechanisms of TCR-MHC/peptide signaling to mediate selection, it is perhaps not surprising that no clear consensus has emerged as to the role of CD28/B7 family members in thymic selection.

As mentioned previously, at least one study has demonstrated that an APC-derived costimulatory signal was required for negative selection of DP thymocytes (71), although the authors failed to identify molecule involved. In vitro studies utilizing antibodies against LFA-1 and ICAM-1 to block the differentiation of double negative (DN) thymocytes (77) or the deletion of DP thymocytes (78) suggest that these molecules may play a role in thymic development. Other molecules implicated in thymic selection include HSA and CD45 (22).

Table 2 Summary: Examples of gp39-dependent and -independent negative selection

Model system	Antigen source[a]	gp39[b]	Effect on negative selection
vSAg (Balb/c)	Endogenous	Anti-gp39 blockade	Blocked
vSAg (CBA/J)	Endogenous	gp39 knockout	Blocked
SEB (Balb/c)	Exogenous	Anti-gp39 blockade	Normal
H-2A[c] (PCC TCR Tg)	Endogenous	Anti-gp39 blockade	Blocked[c]
PCC (PCC TCR Tg)	Endogenous	Anti-gp39 blockade	Blocked
PCC peptide (PCC TCR Tg)	Exogenous	Anti-gp39 blockade	Normal

[a]Indicates whether the selecting Ag/peptide source is endogenous or exogenous (administered i.v).
[b]Indicates the means by which gp39-CD40 interactions were prevented. Anti-gp39 blockade refers to in vivo administration of MR1 (anti-gp39 mAb).
[c]Negative selection was inhibited by > 50% in this system.

5.2 *Evidence for a Role of gp39 in Negative Selection in the Thymus*

Although the respective roles of a number of costimulatory molecules in thymic selection have been investigated, the role of gp39/CD40 interactions was not examined until recently. However, the observation that CD40 is expressed on a wide variety of cell types potentially involved in selection of thymocytes, coupled with the recently documented role of gp39/CD40-mediated regulation of APC costimulatory molecule expression, provides compelling circumstantial evidence that this receptor-ligand pair may regulate some processes of T cell repertoire selection. To test whether signals delivered as a consequence of gp39 engagement were essential for selection of thymocytes, the deletion of T cells to a variety of Ags in both TCR Tg and non-Tg models of negative selection was investigated in vivo. Results of these studies are discussed in more detail below and summarized in Table 2 (21).

5.3 *Negative Selection Mediated by SuperAgs (SAgs)*

Deletion of thymocytes as a consequence of TCR interaction with SAgs, either minor lymphocyte stimulating (Mls) determinants encoded by endogenous mouse mammary tumor viruses (MMTV), or exogenously administered bacterial SAgs such as Staphylococcus Enterotoxin B (SEB), has been used extensively as a model for negative selection (79). Both Mls and SEB have been categorized as SAgs because these Ags are recognized by a large percentage of thymocytes utilizing specific TCR Vβ elements. Studies examining the role

of gp39 in a viral SAg (vSAg)-mediated model of negative selection demonstrated that blockade of gp39-CD40 interactions through the use of neonatally administered anti-gp39 mAb prevented deletion of TCR Vβ specificities typically observed in this system (80). Similar results were found when examining vSAg-mediated deletion in gp39-deficient mice, providing strong suggestive evidence for the requirement of gp39 in negative selection mediated by endogenously expressed SAgs. However, thymocyte deletion resulting from the exogenous administration of SEB was unaltered by the presence of anti-gp39. These results suggest that gp39 may play a role in the process of negative selection, only, however, when SAg is expressed endogenously and presumably at more physiologic concentrations. Although these results support a role for gp39-CD40 interactions in clonal deletion to endogenously presented Ag, thymocyte deletion as a result of TCR recognition of vSAg may be unique from deletion mediated by more conventional forms of endogenous peptide Ags presented in thymus (79). Therefore, the effect of blockade of gp39 function was also examined in several additional Ag-specific models of negative selection.

5.4 *Negative Selection in PCC TCR Tg Mice*

Negative selection was examined in three distinct systems using TCR Tg mice specific for pigeon cytochrome c (PCC) in association with H-2E^k (81). Thymocytes from these mice undergo efficient maturation in the presence of H-2E^k; however, in the presence of H-2A^s, the vast majority of Tg DP thymocytes undergo negative selection, leading to a virtual absence of TCR Tg CD4$^+$ SP thymocytes (82). Blockade of gp39-CD40 interactions during neonatal development in H-2A^s PCC Tg mice dramatically inhibited the clonal elimination of DP thymocytes in these Tg mice. Similarly, clonal deletion that results when PCC TCR Tg thymocytes mature in the presence of an endogenously encoded Tg expressing PCC in the thymus (S Ohen, S Hedrick, manuscript in preparation) was completely prevented if gp39 function was blocked (80). These results provide additional support for the tenet that gp39 is required for negative selection of thymocytes recognizing endogenously expressed Ags. Finally, negative selection of Tg thymocytes can also be achieved by exogenous administration of PCC peptide. This model of clonal deletion, however, was shown to be independent of a requirement for gp39, perhaps because extremely high concentrations of Ag are required to mediate clonal deletion. In addition, these results are consistent with those in the SEB deletion model where anti-gp39 did not alter thymocyte deletion to an exogenously administered Ag.

5.5 *Mechanism of gp39 Involvement in Negative Selection*

It is clear from the experiments summarized in Table 2 that not all models of negative selection are dependent upon gp39-CD40 interactions. However,

in four of the six model systems examined, clonal deletion was dramatically altered by the functional blockade or deletion of gp39. Furthermore, these results showed that in contrast to negative selection mediated by exogenously administered Ag/SAg that appeared to be gp39 independent, negative selection mediated by endogenously expressed Ag/SAg was gp39 dependent. It may be that clonal deletion that occurs following administration of supraphysiological concentrations of Ag/SAg overrides any requirement for gp39-mediated signals. Gp39-dependent selection may also be related to the affinity/avidity of the interactions between TCR and MHC/Ag. In both models of vSAg-mediated deletion, mature Ag-specific T cells proliferate poorly in response to Mls, suggesting that the avidity of the TCR for Mls is weak (83, 84). This is also true of PCC TCR Tg T cells stimulated with H-2As (82). Therefore, it is likely that thymocytes bearing these TCRs also interact with Ags/SAgs in a low affinity/avidity fashion. One could postulate that gp39-CD40 interactions provide additional costimulatory signals necessary to facilitate the deletion of thymocytes bearing TCRs of low affinity/avidity. In contrast, gp39-CD40 interactions may not be required for deletion mediated by high-affinity TCR ligands such as high dose PCC peptide and SEB, which elicit robust proliferative responses from mature T cells.

Although studies are in progress to determine the mechanism by which gp39 facilitates negative selection, the three most obvious possibilities are discussed here and depicted in Figure 3. The first possible mechanism is that

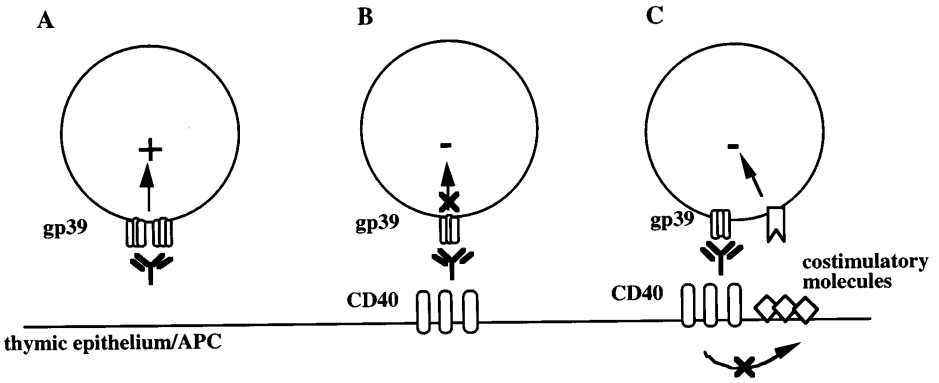

Figure 3 Models for the mechanism of anti-gp39 cross-linking of negative selection. (*A*) A positive signal delivered by mAb cross-linking of gp39 rescues thymocytes from apoptosis; (*B*) A negative signal normally delivered by gp39 engagement of CD40 on thymic APC is blocked by anti-gp39; (*C*) TCR recognition of antigen induces gp39, which in turn engages CD40 and induces the upregulation of costimulatory molecules, which induces a signal causing thymocyte death.

the engagement of gp39 by the mAb delivers a positive stimulatory signal to thymocytes that rescues them from deletion. However, the fact that genetic deletion of gp39 also alters the selection of Vβ-bearing thymocytes by vSAg strongly argues that the absence of gp39 function, and not positive signaling via gp39, alters negative selection. In addition, if this were the case, one might predict that anti-gp39 administration would actually enhance positive selection rather than prevent negative selection. Studies examining the effects of anti-gp39 on positive selection in PCC TCR Tg mice, however, have revealed that positive selection is unaltered as a result of anti-gp39 administration (D Page, S Hedrick, unpublished observations). Finally, although one report does indicate that gp39 engagement can costimulate the activation of mature T cells to anti-CD3 (85), no other reports have ever documented any positive effects of anti-gp39 on thymocytes or mature T cells.

A second possible mechanism may be that blockade of gp39 interaction with its receptor (or absence of gp39) prevents delivery of a negative signal to thymocytes and thus allows their survival. Again, although it is tempting to speculate on a direct role for gp39 signaling thymocytes as a consequence of CD40 engagement following TCR-MHC/Ag recognition, no data exist to support such a signal being transduced via gp39.

The third possible mechanism consistent with the observation of gp39 involvement in negative selection is that gp39 signaling through CD40 regulates the expression of a costimulatory molecule on thymic APCs or epithelial cells that is essential for deletion. Because a major function of gp39 is the regulation of costimulatory molecules (39, 49, 50, 86), it is reasonable to suggest that gp39 regulates negative selection via its influence on costimulatory molecule expression within the thymic microenvironment. Direct evidence that gp39 regulates the expression of B7.2 was provided by the observation that B7.2 expression in the thymic medulla was substantially reduced in gp39 knock-out mice and in mice treated with anti-gp39 (21). Whether the reduced B7.2 is causally related to the alterations in negative selection is currently being evaluated.

5.6 *Implications for Autoimmunity*

There are multiple genetic loci that contribute to an increased risk of autoimmune disease. Mutations in any genetic loci that alter the function of the gp39-CD40 costimulatory "loop" identified within the thymus may alter the efficiency with which self-reactive T cells are centrally deleted and may permit the escape of autoreactive T cells to the periphery. Based on our studies, low-to-moderate affinity, self-reactive T cells would most easily escape deletion when gp39-CD40 interactions are blocked or incapacitated by mutation. However, in the context of the same mutations, high-affinity, self-reactive T cells may be effectively deleted by a gp39-independent mechanism. Xenobiotics, in additions

to mutations, may be autoimmunogenic if they interfere with effective gp39 signaling. Cyclosporine (CsA), an inhibitor of gp39 expression (37, 44), can block negative selection (87, 88). The fact that negative selection is blocked by CsA may be the basis for the increased incidence of autoimmune disease in CsA-treated patients (89–91).

6. THE ROLE OF GP39 IN PERIPHERAL T CELL TOLERANCE

Antigen presenting cells must provide an array of signals to elicit maximal T cell growth, differentiation, and lymphokine production. While presentation of antigen in the context of MHC-encoded molecules is paramount, costimulatory molecules play a critical role in providing additional signals essential for T cell activation. A paradigm of T cell activation is founded on the principle that the T cell must receive at least two signals from the antigen-presenting cell (92, 93). The first signal is engagement and cross-linking of the T cell receptor (TCR) by Ag:MHC, and the second signal is generated through costimulatory signals delivered by other cell surface proteins. Recently, emphasis has been placed on CD28/CTLA4 on the T cell, which binds to B7 family members on the APC, as an important ligand-receptor pair in costimulation (94, 95). Growing evidence indicates that interfering with these costimulatory signals causes negative signaling of the antigen-activated T cells and results in T cell anergy.

6.1 *The Role of CD28/CTLA4 in Tolerance to Alloantigens*

Compelling evidence for the importance of CD28 stimulation in the induction of T cell tolerance to alloantigen or xenoantigen has come from several in vivo model systems. It has been reported that blockade of CD28 signaling by CTLA4-Ig allowed the survival of long-term xenogeneic islet grafts (96). The results showed that CTLA4Ig treatment posttransplantation could lead to prolonged graft survival and long-term, donor-specific tolerance since secondary grafts were not rejected. In addition, Turka et al found that injection of CTLA4Ig enhanced the mean survival time of heart allografts in rats; however, most grafts were eventually rejected (97). In subsequent studies, they found that a donor-specific transfusion of cells at the time of transplant and a single injection of CTLA4Ig on day 2 led to pronounced tolerance (98). Animals receiving this treatment showed long-term graft survival, delayed responses to donor-type skin grafts, and the ability to accept a second donor-specific cardiac allograft. Similar studies have been done in mouse model systems looking at the survival of cardiac allografts with CTLA4-Ig treatments. Pearson et al found that a 12-day course of treatments initiated at the time of transplant produced

indefinite cardiac allograft survival; however, this treatment did not enhance the survival of allogeneic skin grafts (99). They found that recipients with long-term graft survival had donor-specific tolerance because donor-type skin grafts were accepted and third party skin grafts were rejected normally. Although CTLA4Ig treatment was not able to induce tolerance to skin grafts over complete allogeneic disparities, it did cause tolerance to skin grafts with multiple mismatches in minor histocompatibility antigens (100). Taken together, these data strongly suggest that tolerance to alloantigens can be effectively induced by the administration of CTLA4-Ig.

Although a convincing argument for B7-CD28 interactions can be made for the induction of allospecific tolerance, less convincing evidence is available for tolerance to protein or peptide antigens. Although CTLA4Ig has been shown to inhibit T dependent B cell responses (101, 102), antibody responses could be recovered by repeated challenges with antigen, indicating that tolerance was not absolute in these animals. Furthermore, in a recent study, Wallace et al (103) have shown that T and B cells from mice treated with CTLA4Ig were responsive when transferred to irradiated hosts, indicating that the cells had not been irreversibly anergized or deleted. When they investigated the mechanism of suppression induced by CTLA4Ig, they found altered induction of IL-2 and IL-4 mRNA transcripts. Therefore, it is not clear at this time why alloantigen-induced tolerance is more readily attainable compared to tolerance to soluble proteins, but the answer may lie in the characteristics of the APC in the two different situations.

6.2 *gp39-CD40 Interactions in Tolerance to Alloantigens*

Given the compelling evidence that CD28-B7 interactions are critical in allospecific tolerance, we sought to evaluate if gp39, by virtue of its ability to regulate B7 expression, could also induce allotolerance. It has been proposed that resting B cells, which are deficient in the expression of B7.1 and B7.2, are tolerogenic, because they are unable to provide T cells with a "second" signal. A limited number of studies have demonstrated that B cells can induce peripheral T cell tolerance to male antigen (104) as well as soluble antigens (105). The general application of B cells as vehicles to induce peripheral T cell tolerance has not been widely observed. The administration of highly purified, resting allogeneic B cells does not induce a state of allospecific tolerance (across major MHC barriers) that will permit successful engraftment of allogeneic tissue into the treated recipient. We speculated that the reason that the resting B cells were not tolerogenic was that the B cells became activated once they were introduced into the allogeneic host (see Figure 4). Thus, activation of the B cells was due to recognition by host alloreactive T cells that triggered the allogeneic B cells via CD40. As a result of this activation, the allogeneic B cells matured to

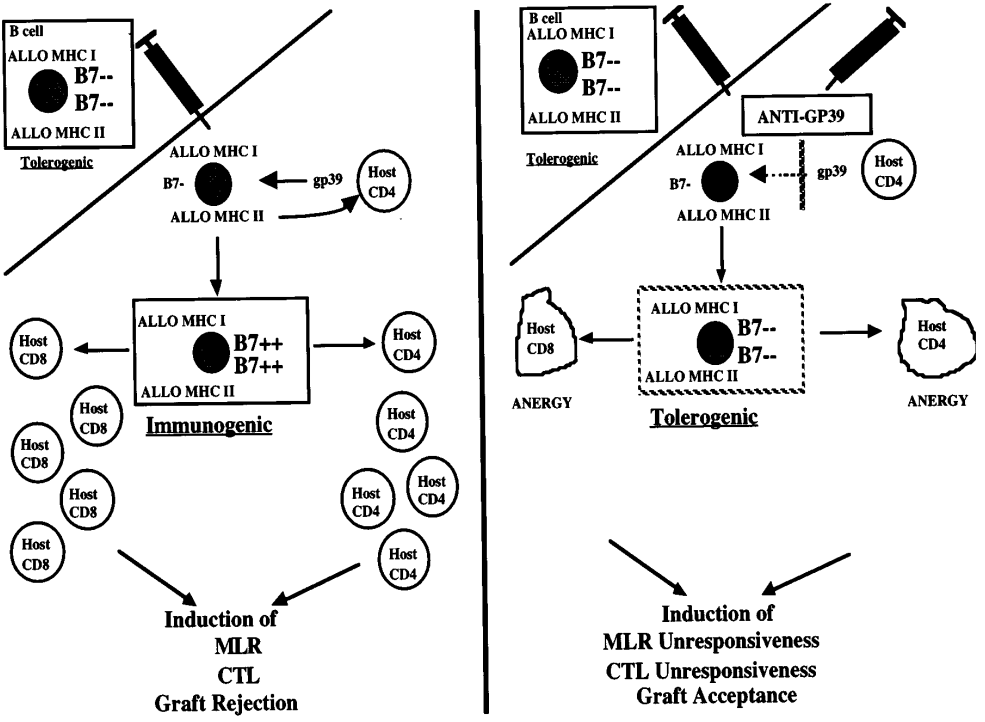

Figure 4 A scheme of anti-gp39-induced peripheral T cell tolerance. See text for details.

"professional" APC and immunized the host against alloantigen. We reasoned that if transferred allogeneic B cells were prevented from becoming activated in vivo by the coadministration of anti-gp39, the allogeneic resting B cells would induce alloantigen-specific tolerance. A recent study (106) showed that administered allogeneic B cells primed both the MLR and CTL responses of the host. In contrast, when coadministered with anti-gp39, the allogeneic B cells induced alloantigen-specific tolerance by measurement of MLR and CTL responses to the relevant alloantigen. Importantly, allogeneic responses to third party alloantigens were unaffected in the tolerized mice. Consistent with these results is the observation that the injection of allogeneic B cells into gp39 knockout mice also induced alloantigen tolerance, confirming that the anti-gp39 mAb is not negatively signaling or deleting the alloreactive T cells. Furthermore, proof that blockade of B cell activation is a critical component of this phenomenon comes from the observation that activation of allogeneic B cells in vitro prior to coadministration with anti-gp39 ablates their tolerogenicity. In summary, it

appears that activation via CD40 is a critical determinant in whether B cells are immunogenic or tolerogenic. Whether the key elements regulated by CD40 tip the balance between immunity and tolerance are B7.1 or B7.2 is unknown at this time.

6.3 *The Induction of Transplantation Tolerance by Interrupting gp39-CD40 Interactions*

Given these results, a model of allograft rejection was used to evaluate whether transplantation tolerance could be induced by the coadministration of allogeneic B cells and anti-gp39. David Parker et al have shown that pretreatment with allogeneic B cells and anti-gp39 can greatly enhance the survival of allogeneic islet grafts (107). In control-treated animals, islet grafts were quickly rejected, and histological examination showed leukocytic infiltration of the grafts. In sharp contrast, the animals that had been treated with B cells and anti-gp39 had > 90% graft survival through the duration of the experiment, and histological examination showed very little leukocyte infiltration of the grafts. These findings are reminiscent, in part, of donor-specific transfusion tolerance (DST) trials, where the infusion of whole blood from the donors induced longer acceptance of donor allografts. DST may be effective if the infused APC are not adequately "activated" by the host maximally to upregulate costimulatory molecules. The combination of DST and anti-gp39 may prove to be a very attractive preparative regime in the allotransplantation.

6.4 *Tolerance Induction During Graft-vs-Host Disease by Interrupting gp39-CD40 Interactions: A Way to Look at Overall Host APC Function in the Absence of CD40 Signaling*

Previous studies clearly indicated that B cells were tolerogenic in the absence of a CD40 signal. Studies were then initiated to evaluate whether blocking the CD40 signal influenced the ability of other APC to stimulate alloantigen-induced responses. Both acute and chronic forms of GVHD are dependent upon T cell activation for initiation and progression of disease. Using the transfer of parental T cells into nonirradiated F1 mice, Durie et al showed that treatment with anti-gp39 at the time of cell transfer could block the development of both chronic and acute forms of GVHD (108). A limited treatment time of 1 week with anti-gp39 prevented the appearance of GVHD for the duration of the experiment. This result suggested that the donor parental T cells were being tolerized in the anti-gp39-treated host. Indeed, transfer of T cells from GVHD mice treated with anti-gp39 to naive F1 mice could not transfer GVHD. This suggests that in the presence of anti-gp39, the host APC could not costimulate

the alloreactive donor T cells and they were rendered tolerant. Supportive data come from the observation that T cells from gp39 knockout mice also cannot mediate GVHD (JE Buhlmann, unpublished data).

Although the mechanisms of anti-gp39-induced allotolerance are unclear at this time, some parallels can be drawn with experiments done in the CD28/CTLA4 system. Initial studies showed that CTLA4Ig was able to reduce acute GVHD-induced lethality in a fully disparate model of GVHD (109). Further studies showed that CTLA4Ig alone was able to protect partially against acute GVHD-induced lethality across multiple minor histocompatibility differences (110). Since CTLA4Ig was not sufficient to block this model of GVHD completely, additional studies were undertaken to see if blockade of alternate signaling pathways could have a synergistic effect. Blazar et al found that hyporesponsiveness could be induced in vitro with CTLA4Ig alone or in combination with anti-LFA1, but when these cells were transferred in vivo, they were still able to induce GVHD (111). When they continued the blocking antibody treatment in vivo after transfer of the cultured cells, they found that the combination of CTLA4Ig and anti-LFA1 was highly effective at preventing acute lethal GVHD across complete MHC differences. Because the GVHD systems used to study the role of CD28/B7 and gp39/CD40 were different, it is hard to make direct comparisons. However, collectively, the data suggest that B7 is important in eliciting GVHD responses and that blockade of CD40 may prevent upregulation of B7 on APC that trigger GVHD. In a more general sense, the data strongly indicate that the donor T cells have to activate the host before the host can trigger an alloreactive T cell response. Which host cells are being triggered by CD40 to elicit the alloreactive response is presently unknown.

7. CONCLUDING REMARKS

Recent studies identifying novel CD40 binding proteins and the biochemical events associated with CD40 signal transduction as well as the studies in molecular modeling of CD40 and its ligand have advanced our understanding of CD40 biology immensely. These breakthroughs along with the numerous in vivo studies demonstrating a clear role for this receptor-ligand pair in the regulation of humoral immunity, antigen-presenting cell function, and central and peripheral T cell tolerance have facilitated our understanding of the multifaceted role CD40 plays in immunity.

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RECEPTORS FOR HLA CLASS-I MOLECULES IN HUMAN NATURAL KILLER CELLS

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KEY WORDS: NK cells, MHC class I, regulatory receptors, cytolytic activity, cytolytic T lymphocytes

ABSTRACT

Natural killer cells are likely to play an important role in the host defenses because they kill virally infected or tumor cells but spare normal self-cells. The molecular mechanism that explains why NK cells do not kill indiscriminately has recently been elucidated. It is due to several specialized receptors that recognize major histocompatibility complex (MHC) class I molecules expressed on normal cells. The lack of expression of one or more class I alleles leads to NK-mediated target cell lysis. During NK cell development, the class I–specific receptors have adapted to self–class I molecules on which they recognize epitopes shared by groups of class I alleles. As such, they may fail to recognize either self-molecules that bound unusual peptides or allogeneic class I molecules unrelated to self-alleles. Different types of receptors specific for groups of HLA-C or HLA-B alleles have been identified. While in most instances, they function as inhibiting receptors, an activating form of the HLA-C-specific receptors has been identified in some donors. Molecular cloning of HLA-C- and HLA-B-specific receptors has revealed new members of the immunoglobulin superfamily with two or three Ig-like domains, respectively, in their extracellular portion. While

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the inhibiting form is characterized by a long cytoplasmic tail associated with a nonpolar transmembrane portion, the activating one has a short tail associated with a Lys-containing transmembrane portion. Thus, these human NK receptors are different from the murine Ly49 that is a type II transmembrane protein characterized by a C type lectin domain. A subset of cytolytic T lymphocytes expresses NK-type class I–specific receptors. These receptors exert an inhibiting activity on T cell receptor–mediated functions and offer a valuable model to analyze the regulatory mechanisms involved in receptor-mediated cell activation and inactivation.

1. INTRODUCTION

Natural killer cells were originally described on a functional basis according to their capability of killing certain tumor cells of hemopoietic origin in the absence of a priori stimulation (1, 2). Since NK cells can lyse target cells that express either syngeneic or allogeneic major histocompatibility complex (MHC) molecules, and even target cells that lack MHC expression, their cytolytic activity has often been referred to as "non MHC restricted" (3–6). It is generally accepted that NK cells provide a first line of defense against certain tumors or viral infections (1, 7). NK cells do not express conventional receptors for antigen, i.e. surface immunoglobulins or T cell receptors (TCR) (8). Their maturation can occur in the absence of a functional thymus in both mice and humans (9, 10). However, recent data indicate that cell precursors capable of differentiating toward NK cells are present in immature thymocytes (11), and a common progenitor for T and NK cells has recently been identified (12, 13). Triggering of NK cells results not only in the induction of their cytolytic activity, but also in the production of cytokines that can exert a regulatory role in the immune response, inflammation, and hematopoiesis (14, 15).

The ability of NK cells to detect and lyse tumor but not normal cells has been interpreted to reflect the existence of multiple receptors for an altered pattern of ligand molecules on tumor cells. These poorly defined NK cell functions and receptors were considered to be homogeneously expressed by virtually all NK cells rather than to be distributed in a clonal fashion. Over the past several years, however, a number of major advances have occurred in our understanding of the functional properties of NK cells, in particular of the mechanisms by which NK cells lyse, or fail to lyse, given target cells. Thus, a number of putative receptors potentially involved in NK cell triggering have been identified. More importantly, an inverse correlation has been established between the expression of surface MHC class I molecules on target cells and their susceptibility to NK cell–mediated lysis. This suggested that MHC molecules

could exert a protective role sparing normal cells from NK cell–mediated lysis. Lack of expression, or masking, of self-MHC molecules, as it may occur in virus infected or tumor cells, would result in susceptibility to NK cell–mediated lysis.

These concepts have been proposed by Klas Kärre and coworkers in the "missing self" hypothesis (16–18). This hypothesis has now been confirmed and extended by experimental data in both mouse and human. In addition, the success in cloning NK cells in humans has been basic for the demonstration that NK cells display a clonal heterogeneity in their ability to recognize given MHC molecules. This finding led to the new concept that NK cells recognize allelic forms of MHC molecules via clonally distributed receptors.

In this review, we focus mainly on the human NK receptors for HLA class I molecules, on their molecular characteristics, and on the functional outcome of the receptor/HLA interactions.

2. MHC RECOGNITION BY NK CELLS

Since their discovery, it has become clear that NK cells can kill target cells that do not express surface MHC class I molecules. Kärre et al suggested that NK cells kill these target cells because they do not express self-MHC molecules (19–21). Thus, according to the Kärre's "missing self" hypothesis (16), NK cells can detect and eliminate target cells that do not adequately express normal self-MHC molecules. Indeed, target cells susceptible to NK cell–mediated lysis are characterized by a defective expression of one or more MHC class I alleles. This situation is typical of some virus-infected or tumor cells that either downregulate certain class I alleles or express an altered peptide pattern presented by MHC class I molecules (22, 23). In the context of the "missing self" hypothesis, Kärre & Ljunggren hypothesized the existence of NK cell receptors specific for MHC class I molecules that would deliver a "negative" signal resulting in inhibition of the NK cell cytotoxicity. Indeed the role of the interaction between MHC molecules and the putative MHC-specific NK receptors would be confined to the (negative) regulation of a number of activating signals delivered upon recognition of non-MHC-encoded ligands (24, 25). It is evident that NK cells appear complementary to cytolytic T cells that are triggered by class I proteins presenting foreign (e.g. viral or tumor) peptides. Since some virus-infected or tumor cells downregulate the expression of class I proteins and can thus escape T cell surveillance (26–29), the NK cells are likely to play an important role in the host defense by eliminating these MHC-negative cells.

The "missing self" hypothesis does not exclude the possibility that normal cells expressing adequate levels of MHC class I molecules can also be killed

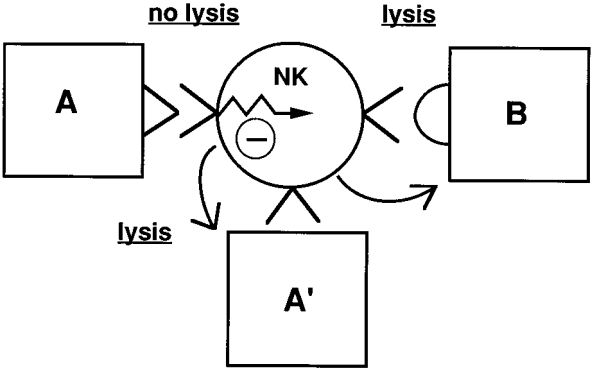

Figure 1 NK cells do not lyse self-normal cells (*A*) but lyse some allogeneic cells (*B*) or self-cells that do not express a protective HLA class I allele (*A′*). Each NK cell expresses at least one inhibiting receptor specific for a self-class I allele. As a consequence, they do not lyse normal autologous cells (*A*). However, autologous cells, as a consequence of viral infection or tumor transformation, may lack the expression of one or more class I alleles (*A′*). Likewise, cells may express altered class I molecules, e.g. because they bind abnormal peptides derived from viral proteins. In both cases these cells become susceptible to NK-mediated lysis. Allogeneic cells that express MHC class I alleles unrelated to self-alleles are lysed because no efficient inhibiting interactions between NK receptors and class I molecules can occur (*B*).

by NK cells. This, however, occurs only with certain allogeneic cells carrying a mismatched MHC haplotype (30, 32). In addition, other non-MHC ligands may affect the susceptibility to NK cells. Indeed, some MHC-negative cells may be resistant to NK-mediated cytotoxicity. In this case, target cells probably lack appropriate ligands for adhesion or for triggering the activating NK receptors (24). It should be stressed, however, that killing of autologous cells with a normal expression of MHC class I has never been observed, and that loss of MHC class I expression is sufficient to render them susceptible to NK cell–mediated lysis (33). This has been unequivocally shown by the analysis of TAP$^-$ or β2m-deficient target cells (34, 35). These data imply that NK cells adapt their receptors to their MHC haplotype so that they can recognize and be inhibited by self-MHC alleles. It should also be noted that certain allogeneic MHC class I alleles may function as protective elements. As we discuss below, the protective non-self-alleles share with self-alleles particular amino acid sequences recognized by the NK receptors (36, 37) (Figure 1).

2.1 *Murine Models of MHC–NK Cell Interactions*

Genetic control for NK cell–dependent rejection of bone marrow or tumor grafts in the mouse maps to the H-2D or -K regions (38). Introduction of bone

marrow cells from β2m-negative or -positive H-2^b mice into allogeneic (H-2^d) mice leads to the rejection of both types of grafted cells; however, a preferential rejection of β2m-negative allogeneic cells was observed, suggesting that at least some allogeneic MHC class I molecules may protect from NK cell–mediated cytotoxicity (39). Direct evidence of the role played by MHC alleles in shaping the NK cell receptor repertoire has been obtained in mice transgenic for a given MHC class I product and in knockout mice that did not express MHC class I molecules. Introduction in the B6 mouse (H-2^b) of a novel MHC class I allele (H-$2D^d$) as a transgene resulted in NK-mediated rejection of syngenic grafted cells from nontransgenic mice (36).

Interestingly, the rejection of target cells from β2m or TAP-1 deficient mice, which usually occurs in a normal host (35), is abrogated when the host carries the corresponding defects (40–42). In this case no self-MHC class I molecules should be available for shaping the NK receptor repertoire; however, mice were found to express low levels of β2m-free or peptide-free MHC class I molecules, so that both T and NK cells might be educated on these peculiar phenotypes (43).

2.2 *MHC-NK Cell Interaction in Humans*

Analysis of the MHC recognition by NK cells in humans has been performed primarily by the use (as target cells) of in vitro cultured tumor cells, PHA-induced T cell blasts, and cells transfected with HLA genes. The use of HLA class I–deficient cell lines transfected with different HLA class I genes demonstrated the protective role exerted by these molecules from NK-mediated lysis. In early studies polyclonal NK cell populations were used as effectors. These cell populations displayed a marked increase in the ability to lyse cell variants lacking the expression of HLA class I molecules (33, 44–47). Transfection of these cell variants with different HLA class I gene products gave controversial results regarding their ability to protect target cells from NK cell–mediated lysis (48, 49). For example, transfection of single HLA class I genes in a small-cell lung carcinoma or in K562 and MOLT-4 cell lines did not result in a consistent protective effect. On the other hand, transfection of the β2m gene into the β2m-negative Daudi cell line resulted in the reexpression of HLA class I and in a sharp decrease in susceptibility to NK-mediated lysis (50). Similar data were obtained after transfection of C1R cells (a cell variant expressing Cw4 as the only serologically detectable class I allele) (33, 51) with HLA-A3, HLA-B7, or HLA-B58 alleles (52). β2m-transfected Daudi cells were resistant not only to freshly isolated NK cells, but also to IL-2-activated NK cells (LAK cells). On the contrary, C1R transfectants expressing an incomplete MHC haplotype (i.e. Cw4 and the transfected gene), were susceptible to IL-2-activated NK cells. An

explanation for these data has been provided in subsequent studies in which we showed that NK cells are heterogeneous in their ability to recognize different HLA-class I alleles.

2.3 *Cloning of Human NK Cells and Definition of a NK Cell Repertoire*

A major breakthrough in understanding the NK cell heterogeneity was provided by the development of culture conditions allowing cloning of NK cells (53). NK cell clones derived not only from different donors but also from single donors were found to display different patterns of cytolytic activity against a series of normal allogeneic cells (PHA-blasts). Thus, each group of clones displayed a unique pattern of cytolytic activity against the allogeneic donors. These data suggested the existence of different groups of NK cell clones characterized by different specificities (18, 32, 54, 55). It is also noteworthy that in no instances did NK clones lyse autologous PHA blasts. These experiments provided evidence for the existence of a functional NK cell repertoire related to the ability to lyse allogeneic cells (24, 32). Also data reported by Bender et al (56) on the lysis of allogeneic endothelial cells could be interpreted on the basis of the existence of (at least) two distinct NK specificities in a single donor. In our studies, four NK-defined specificities have been determined in a single donor; however, $> 60\%$ of NK clones isolated from this donor did not lyse any target cell in the panel (32).

In view of these results, our next goal was to identify the target molecules responsible for the susceptibility/resistance to lysis. The availability of alloreactive NK cell clones displaying different cytolytic patterns made possible a segregation and genetic analysis of the characters' susceptibility or resistance to lysis by different NK clones. In these experiments, PHA-blasts isolated from different members of representative families have been used as target cells. This analysis provided evidence that the character resistance to lysis cosegregated with certain HLA haplotypes (54, 55). For example, the character resistance to lysis by NK cell clones belonging to group 2 (32) cosegregated with the Cw3 allele. On the basis of these data, we hypothesized that the expression of the Cw3 allele could protect target PHA-blasts from lysis by group 2 NK clones. At that time, evidence already existed concerning the possible protective effect exerted by MHC class I molecules on target cells; however, no evidence suggested that allelic forms of MHC molecules could specifically protect target cells from lysis by given NK cells, i.e. that a clonal heterogeneity existed in the ability to recognize allelic forms of MHC molecules. Further suggestive evidence that this could indeed be the case was obtained by the selection of Cw3-negative cell variants from a $Cw3^+$ EBV cell line (57). All cell variants lacking Cw3 had acquired susceptibility to lysis selectively by group 2 NK clones.

2.4 *HLA–Class I Alleles as Ligands for Specific Recognition Mediated by Human NK Clones*

The direct demonstration that HLA class I alleles can function as specific protective elements against defined groups of NK clones was provided by the analysis of murine P815 cells transfected with different human HLA class I alleles. Initially, we focused primarily on the Cw3 allele. Transfection of murine P815 cells with the human gene coding for Cw3 molecules conferred resistance to lysis to the otherwise sensitive P815 cells (57). The protective effect was confined to group 2 NK clones. P815 cells transfected with other class I alleles including A2, A3, and A24 did not confer resistance to group 2–mediated lysis.

Further analysis of other HLA-C alleles indicated that Cw4 acts as a protective element for group 1 NK clones (57). This was also shown by the use of the C1R cell line, which expresses Cw4 as the only serologically detected class I molecule. With the exception of group 1 NK clones, C1R cells were efficiently lysed by clones displaying various specificities (58, 59).

Analysis of representative families suggested that in addition to Cw3 or Cw4, other HLA-C alleles could function as protective elements from lysis mediated by either group 1 or group 2 NK clones. Thus, in the case of group 1 clones, the protective alleles included Cw2, Cw4, Cw5, and Cw6, while in the case of group 2 clones, the protective alleles were represented by Cw1, Cw3, Cw7, and Cw8 (60). By the comparative analysis of the amino acid sequences of different HLA-C alleles, we and others showed that those recognized by group 1 NK clones share the Asn 77 and Lys 80 in the peptide binding groove (α1 domain) of HLA-C molecules. On the other hand, the HLA-C alleles recognized by group 2 NK clones are characterized by Ser 77 and Asn 80 (61, 62). The direct evidence of the critical role of these amino acid residues in determining the specific recognition by group 1 or group 2 NK clones has been provided by experiments of site directed mutagenesis involving residues 77 and 80 (63). The results indicated that single amino acid substitutions at position 77 and 80 in Cw3 and Cw4 molecules are sufficient to alter the protection of target cells from lysis by group 1 and group 2 NK clones. In particular, the Lys 80 and Ser 77 residues are essential to mediate protection from group 1 and group 2 clones, respectively. In addition, a Cw3 mutant molecule characterized by Asn 77 and Lys 80 residues (that are typical of the Cw4 alleles) lost the protective effect typical of Cw3 and acquired that typical of Cw4 molecules. Our results are consistent with the finding by Storkus et al (64) that amino acid substitution at position 74, in the α1 helix of HLA-A2, modified the capacity of this molecule to protect target cells from lysis by polyclonal NK cells obtained from human peripheral blood.

These results, together with data in murine models, are in line with the concept that NK cells may, at least in some instances, sense the combination of

MHC and peptide, possibly because binding of the peptide induces changes in the molecular conformation of MHC molecules.

2.5 *General Role of HLA Molecules in the Protection from NK Cell–Mediated Lysis*

Our studies clearly indicated that HLA-C functioned as protective elements only for a fraction of NK clones (24). It was important to define whether NK clones that were not HLA-C-specific could recognize other HLA class I molecules. This possibility was suggested by the report that peripheral NK cell populations less efficiently lysed MHC class I–deficient target cells transfected with a series of HLA class I molecules including A3, A11, A24, Aw68, B7, and B27 (52). Moreover, our studies on the genetic segregation of characters' susceptibility/resistance to lysis by all groups of clones analyzed indicated that, in all instances, the analyzed character segregated with the HLA haplotypes (32). More direct evidence that indeed all NK cells recognize HLA class I molecules was provided by the use of anti-class I mAbs. In these experiments, selected anti-HLA class I mAbs were shown to reconstitute lysis of resistant target cells by all clonogenic NK cells analyzed (65, 66). A likely explanation was that mAb–mediated masking of HLA class I molecules prevented target cell recognition by NK cells and delivery of a negative signal. The most important information provided by this approach was that class I molecules are directly involved in the mechanism of protection from autologous NK cells, suggesting that self-class I molecules may act as elements that specifically protect normal cells from NK cell–mediated autoreactivity (65, 66).

In view of these findings, the HLA allele specificity of NK clones that were non HLA-C-specific was analyzed against suitable cell lines and cell transfectants. These studies led to the identification of NK cell clones specific for two distinct groups of HLA-B alleles belonging to the serologically defined HLA-Bw4 or Bw6 supertypic specificities (67–70). Comparison of the amino acid sequencies in the $\alpha 1$ domain of HLA-B molecules suggested that isoleucine (Ile) or threonine (Thr) at position 80 correlated with the resistance to lysis by NK clones specific for the Bw4 specificity. Thus, as directly demonstrated for the NK-mediated HLA-C recognition, single amino acid positions appear to be critical for HLA-Bw4 recognition. These Bw4-specific NK cell clones were found to correspond to the previously identified group 3 (32) of NK clones (71).

Further analysis of HLA-defective cell variants transfected with HLA-A alleles revealed the existence of NK clones recognizing some of these class I alleles (72). Although these studies will require the analysis of a large panel of NK clones and of HLA-A transfectants, preliminary data are in line with the concept that NK cells can specifically recognize allelic forms of HLA-A.

Indeed clones have been identified that did not lyse HLA-A3 or A11 while they efficiently killed untransfected cells or cells transfected with HLA-A2.

2.6 *The Role of Class I–Bound Peptides in NK Cell–Mediated Recognition*

An important question regarding inhibition of NK cells by class I molecules on target cells concerns the possible role of class I–bound peptides in determining the specificity of the reaction. As discussed above, single amino acid substitutions in the $\alpha 1$ helix of both HLA-A (64) and HLA-C (63) altered the protective effect of class I molecules. This substitution is thought to modify the population of peptides that can bind to the HLA molecule. These data raised the possibility that NK cells might be inhibited by specific peptide/class I complexes. Noteworthy is that residues 77 and 80 are localized in the peptide-binding groove and, more precisely, contribute to the formation of the F pocket (73). According to Zemmour & Parham, both amino acids have potential contact with a bound peptide (74). In this context, NK cells may recognize directly the bound peptide, or alternatively, they could sense changes in the conformation of MHC class I molecules caused by peptide binding.

It is possible to speculate that the function of these peptides would be to define the autologous cells as normal or abnormal. In the case of viral infection, the normal self-peptides can be displaced or competed with by an excess of peptides derived from viral proteins synthesized in the infected cell (75). This would lead to the inability of NK cells to recognize the new MHC/peptide complexes and would also imply that infected cells could be interpreted by an NK cell as missing self even when they do not have an altered quantitative expression of MHC class I molecules.

Experimental evidence from several different laboratories supports the involvement of MHC-bound peptides in MHC recognition by NK cells (23, 76–78). Two possible roles for peptides in NK cell recognition have been proposed (79). They could be important simply because they bind and stabilize class I molecules, in which case any peptide characterized by a structural motif allowing binding to a given class I molecule would confer protection from NK cells. An alternative model is that only the critical peptides that bind to class I molecules and also provide particular structural motifs that allow recognition by NK cells would exert a protective effect. Recently, by the use of Bw4-specific NK clones, we showed (78) that the simple binding to HLA-B27.05 is not sufficient for a peptide to provide protection. Indeed, only one of several binding peptides conferred protection, thus suggesting that only this particular peptide could provide a self-motif sensed by NK cells. In contrast, in mouse, H-2D^{I-}-specific NK cells could recognize virtually all H-2D^{I-} peptide combinations tested and be inhibited in their cytolytic activity

(80). These discrepancies may be related to differences in the molecular characteristics of the NK receptors that may recognize either the MHC itself or the epitopes formed by given peptides. In the case of peptides conferring selective protection (as in the case of the protective HLA-B27.05-bound peptides), it will be important to analyze the effect of systematic substitutions in their amino acid sequences to reveal the motif influencing the NK cell–mediated recognition.

3. THE NK RECEPTORS FOR MHC MOLECULES

3.1 *The MHC-Specific NK Receptors in Mouse*

In the early 1990s, the putative receptors for MHC molecules were identified in both mouse and humans. In mouse the receptor responsible for MHC recognition is represented by the Ly49A molecules (81). Ly49A is expressed on a subset of C57BL/6 NK cells corresponding to approximately 20% of the total NK cell population. Ly49A$^+$ cells are unable to kill target cells expressing H-2D^d or H-2D^k. This reflects a direct interaction of Ly49A molecules with class I molecules. Indeed the protective effect could be overcome by mAb-mediated masking of either Ly49 A or class I molecules (in particular the ($\alpha 1/\alpha 2$ domain). In addition, a direct demonstration has been provided for binding of Ly49A receptors to purified D^d or D^k molecules (82). Ly49A belongs to a multigene family displaying a high degree of homology. Although it is possible that the various members of the Ly49 family may recognize other class I alleles, the direct demonstration of this specificity has not yet been obtained. Some of these putative receptors have been identified by mAbs including the 5E6 (specific for the Ly49C) (83) and the LGL-1 (specific for the Ly49G) (84). The inhibiting MHC ligand for Ly49C molecules appears to depend on the H-2 type of the host. Thus, Ly49C molecules receive a negative signal from both H-2b and H-2d or from H-2k^b only in two different strains of mice.

Ly49A is a homodimer type II integral membrane protein with COOH-terminal extracellular domains homologous to members of the C-type lectin superfamily. The Ly49 multigene family is encoded by a gene cluster, termed *NK gene complex* (81), which is found on chromosome 6 in association with other genes encoding for proteins (CD69 and NKR-P1) displaying a structure similar to Ly49.

It is important that the Ly49A and Ly49C receptors are each subject to allelic exclusion (85). Since Ly49 genes are not thought to undergo DNA rearrangement, the allelic exclusion could involve a new mechanism different from that used for TCR and Ig.

3.2 *The Human NK Receptors for HLA-C Molecules*

The finding that NK cells express a clonally distributed ability to recognize different MHC class I alleles suggested the existence, on the NK cell surface, of receptors mediating this recognition. Based on the available experimental data the putative receptors for a given class I allele should be expressed by only a fraction of NK cells and should be able to negatively regulate the cytolytic activity of NK cells. In humans, the first molecules displaying these characteristics were identified by two mAbs termed GL183 (86) and EB6 (87). These mAbs reacted with different NK cell subsets, and the expression of the corresponding surface molecules was not altered by cell activation, proliferation, and cloning. In addition, $EB6^+$ or $GL183^+$ cells varied in percentage among different donors. The EB6 or GL183 molecules belong to different members of the same molecular species, termed p58 (88). At the clonal level, a close relationship could be determined between the expression of EB6 and GL183 molecules (indicated as p58.1 and p58.2) and the specificity for different HLA-C alleles (87, 88).

These data suggested that p58.1 and p58.2 molecules could be involved in the recognition of different HLA-C alleles. This concept was further sustained by the finding that soluble anti-p58 mAbs induced lysis of HLA-C-protected target cells by $p58^+$ NK clones (58, 59). A likely interpretation of these data is the occurrence of a mAb–mediated masking of the receptor. This would result in blocking of the MHC/receptor interactions with consequent inhibition of the delivery of the negative signal to NK cells. On the other hand, when cross-linked by FcγR expressed on target cells, anti-p58 mAbs strongly inhibited the cytolytic activity. This inhibition was similar to that occurring when specific p58 receptors interacted with protective MHC molecules. Thus, anti-p58 mAbs bound to surface FcγR may mimic the HLA-C–mediated cross-linking of p58 receptors (58, 59).

Regarding the HLA-C allele specificity of p58.1 and p58.2 molecules, we originally showed that NK clones belonging to group 1 were characterized by the $GL183^-/EB6^+$ (87) phenotype whereas group 2 clones were $GL183^+/EB6^{dull}$ or $GL183^+EB6^-$ (18, 24, 32, 88). Thus, the cytolytic activity of group 1 clones against target cells expressing Cw4 or related alleles (characterized by Asn 77 and Lys 80) was reconstituted by mAb–mediated masking of the p58.1 (EB6) receptor (58, 59), whereas the cytolytic activity of group 2 clones against cells expressing Cw3 or related alleles (characterized by Ser 77 and Asn 80) was reconstituted by mAb-mediated masking of the p58.2 (GL183) receptor (58). These data strongly suggested that p58.1 and p58.2 molecules represented the receptors for two distinct groups of HLA-C alleles.

More recently, we described a group of $GL183^+/EB6^{bright}$ NK clones characterized by the inability to lyse all allogeneic target cells, independent of their

HLA-C haplotype (89). On the other hand, these clones, defined as *group 0*, could lyse HLA-class I-negative target cells while class I^+ target cells were lysed only in the presence of anti-class I mAbs. Unlike group 1 or group 2 clones, the group 0 NK clones appeared to recognize all the HLA-C alleles. Recognition was shown to be mediated by p58.1 and p58.2 molecules, functioning as independent inhibiting receptors for the two distinct groups of HLA-C alleles (which encompass all the expressed HLA-C molecules).

At present it is still difficult to explain why the p58.1 molecules expressed on group 1 and group 0 clones recognize Cw4 and related alleles, while those expressed on group 2 clones do not. A likely explanation is the lower number of p58.1 molecules expressed by the latter clones (89). Thus, while the fluorescence intensity of the staining with anti-EB6 mAbs is high in group 1 and group 0 clones, in group 2 clones it is low. A critical number of p58.1/Cw4 interactions may be required to result in efficient receptor cross-linking and transduction of the (negative) signal. In agreement with this interpretation are data on the mAb–mediated modulation of p58.1 molecules in group 1 clones (59). These experiments showed that modulation (i.e. internalization) of p58.1 molecules resulted in lysis of $Cw4^+$ cells, and that a critical number of p58.1 molecules (re-expressed after modulation) was required to generate an effective negative signal upon recognition of the protective allele. If this interpretation is correct, one may predict that group 2 clones expressing the $GL183^+/EB6^{dull}$ phenotype could recognize with low efficiency (and thus induce a poorly efficient negative signal) HLA-Cw4 and related alleles, i.e. the alleles recognized by conventional p58.1 receptors. It is also possible that this low reactivity of anti-p58.1 mAb may reflect a peculiar characteristic of the p58 molecules expressed by group 2 clones. Indeed p58.2 (GL183) molecules may be heterogeneous and in some cases may express epitopes also recognized by anti-p58.1 mAbs (although with low avidity). This interpretation would explain the finding that anti-p58.1 mAbs could partially reconstitute the cytolytic activity of group 2 clones expressing the $GL183^+/EB6^{dull}$ phenotype (58).

Genes encoding for p58.2 as well as those encoding for p58.1 molecules have recently been identified (see below). It will be important to analyze all transcripts for p58.2 molecules, particularly those derived from $GL183^+/EB6^{dull}$ clones, to define the EB6 mAb reactivity of the encoded proteins.

3.3 *HLA-C Specific NK Receptors Mediating NK Cell Activation*

While the best-characterized NK receptors for MHC class I are the inhibiting receptors, several pieces of experimental evidence in rat (90, 91) and mouse (39, 92) suggested that MHC class I receptors may also deliver triggering

signals. We recently showed that p58.1 (and p58.2) molecules also exist as activating receptors that, upon binding to specific ligands, deliver a positive rather than a negative signal to NK cells (93). As a consequence, different from that of the inhibiting receptors, cross-linking of the activating receptors results in both early events (such as Ca^{++} mobilization) and late effector functions (e.g. triggering of cytolytic activity and of cytokine production). Unlike the inhibiting p58 receptors, the activating receptors were detectable only in some donors. In addition, they were clonally distributed, and in no instances did single NK cells coexpress activating and inhibiting receptors displaying an identical HLA-C specificity. Comparative biochemical analysis of the activating and the inhibiting receptors showed remarkable differences. Thus, the activating receptor, different from the typical 58-kDa receptor, displayed a molecular mass of 50 kDa. This reflects differences in the protein backbones (42 and 36 kDa, respectively) rather than in the degree of glycosylation of the same protein. Treatment with different proteolytic enzymes, followed by peptide mapping, indicated that p50 and p58 molecules belong to the same molecular family (93). These data suggest that the p50 and p58 receptors may be coupled to different pathways of signal transduction, possibly reflecting differences in their cytoplasmic tails characterized by divergent amino acid sequences within this portion of the molecule. Indeed, this hypothesis has recently been confirmed by cloning the p58/p50 molecules-encoding genes (94, 95, 96).

It is of note that all NK clones expressing an activating p50 receptor coexpress another receptor, specific for a different class I allele but characterized by an inhibiting function. Moreover, the inhibiting receptor always downregulated the NK cell activation induced via p50 molecules (93). The predominance of the inhibiting receptor implies that a measurable NK cell triggering via p50 molecules occurs only in the absence of effective inhibiting interactions. Such event would occur in pathological conditions characterized by downregulation or (peptide-induced) alteration of the protective MHC alleles.

Although the activating receptors appear to recognize the same class I allele recognized by the corresponding inhibiting receptors (93), the experimental approach used so far does not allow definition of whether the two receptors can sense different MHC-bound peptides. Indeed one may speculate that triggering of activating receptors may take place also with MHC-peptide interactions that are not recognized by inhibiting receptors.

A third form of activating receptor belonging to the p58/p50 molecular family has recently been identified in our laboratory. This molecule (p58.3) does not react with GL183 or EB6 mAb and is expressed only by some donors. So far, only an activating form of this molecule has been identified (by the use of a specific mAb). Although biochemical analysis indicates a high degree of similarity

with p58/p50 molecules, no evidence for HLA-C specificity has been obtained. Importantly, mAb-mediated triggering of peripheral blood lymphocytes from individuals expressing p58.3$^+$ NK cells induced a selective expansion of these cells. These data suggest that, following receptor–mediated signaling, NK cells can undergo selective proliferation, reminiscent of the T cell proliferation in response to conventional antigens or superantigens.

Whether the MHC-induced triggering of the NK-mediated cytotoxicity can be regarded as a major pathway of NK cell activation remains to be determined. In this context, however, it should be recalled that HLA-negative cells are highly susceptible to the NK cell–mediated lysis. Obviously, in this case, no NK cell triggering can be induced by activating receptor/MHC interactions, and the existence of activating receptors specific for ligands different from MHC molecules must be postulated.

3.4 *Signaling Via HLA-C Specific p58/p50 Receptors*

As discussed above, p58 and p50 molecules deliver opposite signals upon binding to their specific ligands.

The molecular basis of the negative signal is still poorly understood. We showed that p58 molecules form a complex with a member of the tyrosine kinases of the src family (p56^{lck}) and with different members of the ζ family including (at least) the ζ-CD3 and the γ-FcϵRI. However, in contrast to the triggering of the CD3/TCR complex in T cells or of CD16 molecules in NK cells, no tyrosine phosphorylation could be detected in the p58-associated molecules belonging to the ζ-family (59).

Analysis of the p50-associated molecules did not reveal the presence of typical CD3-ζ or Fcϵ-Rl-γ chains. However, while cross-linking of inhibiting p58 molecules did not result in $[Ca^{++}]_i$ increases, the cross-linking of the p50-activating receptor resulted in a strong $[Ca^{++}]_i$ increment (93). Notably, upon mAb–mediated cross-linking of p58 molecules, no inhibition of the early steps of NK-cell activation could be detected (59). Thus, although NK-cell activation induced via p50 or CD16 molecules was completely blocked by the simultaneous cross-linking of p58 molecules, no inhibiting effect was detected on $[Ca^{++}]_i$ mobilization. Our interpretation of these data was that the inhibiting pathway induced via p58 molecules may converge with the activating pathways at a later stage during the signaling cascade and may involve still unidentified downstream transducing elements. By applying a different experimental approach, Kaufman and colleagues showed that MHC class I recognition by inhibiting NK receptors did not inhibit cell adhesion to target cells (97). However, in contrast to our data, their report indicates an inhibiting effect on both $[Ca^{++}]_i$ mobilization and inositol phosphate release (98). It is important to consider that most normal cells in an organism are protected from NK cell–mediated

cytotoxicity because they express class I molecules. Since the NK.R/MHC class I interaction leads to NK cell inhibition, one may ask why NK cells, continuously exposed to inhibiting signals (i.e. self-MHC class I alleles), are still able to lyse bystander, unprotected cells (e.g. tumor cells). On the basis of the available data, a possible explanation is that the inhibiting effect is limited to a portion of the cell. This polarization effect of the negative signal would not prevent NK cells from mediating cytolysis by utilizing other cell surface compartments (88).

3.5 *Molecular Clones of the HLA-C Specific Human NK Receptors*

Yokoyama & Seaman (81) showed that murine Ly49 molecules belong to a family of strictly related type II transmembrane proteins containing a C-type lectin domain. The Ly49 receptor genes were found to cluster to the NK gene complex on mouse chromosome 6. Molecular cloning of p58 human NK receptors was first reported by Wagtmann et al in 1994 (94). These studies revealed a structure completely different from that of Ly49. The p58 molecular family (at least six different homologous genes have been identified) is characterized by two immunoglobulin-like domains in the extracellular portions (95, 96). The finding of two unrelated receptor families serving similar functions in mouse and in humans has several implications. First, it is likely that these receptors may sense differently the MHC class I molecules and their changes. In this context, as referred above, Correa & Raulet showed that H-2D^d recognition by NK cells was dependent upon binding of H-2D^d to specific peptides but could not discriminate among different peptides (80). In contrast, Malnati et al (78) showed that recognition of HLA-B27 by human NK clones was both peptide dependent and peptide specific. The molecular structure of the Bw4-specific NK receptors (i.e. the NKB1/Z27 p70 molecules) has recently been determined. They are represented by an immunoglobulin-related receptor, similar to p58 molecules but displaying three Ig-like domains (99). Clones encoding for p58.1 or p58.2 molecules were found to display 84% sequence homology. Although amino acid differences between the two sequences were scattered over the entire molecule, eleven residue differences in the extracellular portions were typical of either p58.1 or p58.2 molecules. These findings could explain differences both in the specific binding of HLA-C alleles and in the antibody reactivity of the two molecules.

A remarkable feature of p58-related sequences was that molecules that were highly homologous in their extracellular domains expressed different cytoplasmic tails. The long cytoplasmic tail was 76 or 84 amino acids and contained a typical sequence related to the antigen recognition activation motif (ARAM)

present in CD3 molecules (100). The short tail was 39 amino acids, did not contain an ARAM motif, and was associated to a transmembrane region containing a Lys residue (absent in the other form). These findings were highly suggestive of receptors displaying identical specificity but transducing different signals. Biassoni et al analyzed whether the activating p50 forms of the HLA-C specific receptors correlated with molecules characterized by a short tail. Indeed, a precise correlation between a short cytoplasmic tail and an activating p50 form of the receptor was found for both EB6 and GL183-reactive molecules (96) (see Figure 2).

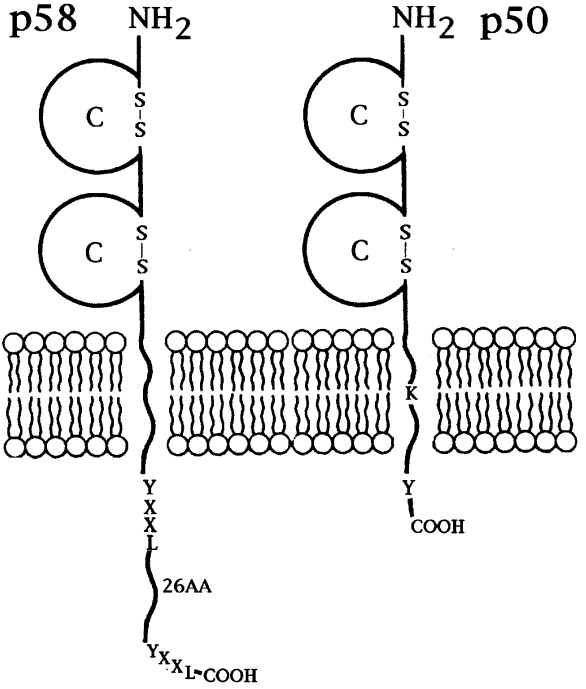

Figure 2 HLA-C-specific NK receptors belong to the Ig-superfamily. Both inhibiting (p58) and activating (p50) receptors are type I transmembrane proteins belonging to the Ig-superfamily. They display two Ig-like domains sharing a high degree of amino acid homology. The inhibiting form (p58) is characterized by a nonpolar transmembrane portion associated with a 76–84 amino acid tail containing an ARAM-like motif characterized by tandem Tyr-X-X-Leu sequences spaced by 26 amino acids. The typical ARAM motifs present in the TCR-associated CD3 chains are spaced by 6 to 8 amino acid residues. In contrast, the activating form (p50) had a 39 amino acid cytoplasmic tail that did not contain the ARAM-like motif due to an in-frame stop codon after the Tyr codon of the first Tyr-X-X-Leu sequence and was always associated to a transmembrane portion containing a charged amino acid residue (Lys).

3.6 *The Human NK Receptors for HLA-B Molecules*

A fraction of NK cells, variable in size in different individuals, does not express p58 molecules as detected by the GL183 or EB6 mAbs and yet recognizes class I molecules on potential target cells (see above). Therefore, the search for putative receptors for HLA-B or HLA-A alleles has been performed in p58-negative NK clones. NK clones shown to express inhibiting receptors for HLA-B alleles were used for mice immunization. The resulting mAbs were screened on the basis of their ability to reconstitute the cytolytic activity of the immunizing clones against HLA-B-protected target cells. When mAbs were raised against Bw6-specific NK clones, all the selected mAbs reacted with a previously identified molecule involved in NK cell functions represented by the CD94 (Kp43) molecule. Anti-CD94 mAbs specifically reconstituted the cytolytic activity of Bw6-specific, but not that of Bw4-specific clones (67). In contrast to p58 receptors, which are restricted to NK cell subsets, CD94 molecules are expressed on the majority of peripheral blood NK cells (101–103), although the CD94 molecules display noticeable variations in the level of expression among different NK cells. We found that Bw6-specific NK clones were confined to the CD94^{bright} subset. In redirected killing experiments against P815 cells, CD94 mAbs of IgG isotype inhibited the spontaneous lysis of target cells by CD94^{bright} clones, thus mimicking the protective effect of HLA-Bw6 alleles (67). In contrast to p58 receptors, which are restricted to NK cell subsets, CD94 molecules are expressed on the majority of peripheral blood NK cells (101–103), although the CD94 molecules display noticeable variations in the level of expression among different NK cells.

More recently, evidence has been accumulated that CD94 molecules are not exclusively involved in the recognition of HLA-B alleles. For example, the cytolytic activity of some NK clones expressing the CD94^{bright} phenotype was found to be inhibited by certain HLA-B alleles belonging to the Bw6 subgroup and by HLA-A alleles including A1, A3, and A11. The cytolytic activity of these clones against both HLA-B- and HLA-A-transfectants could be restored by anti-CD94 or anti-HLA class I mAbs. Although these clones did not express p58 receptors, they did not lyse efficiently 221 cells transfected with HLA-C. Moreover, the lysis of these target cells was reconstituted by anti-CD94 mAbs. However, it is of note that while HLA-C molecules exerted an efficient protection from p58^{+} clones, the protection was only partial in the case of the above p58-CD94^{+} clones. In addition, in p58^{+} clones (which usually express a CD94^{dim} phenotype), anti-p58 but not anti-CD94 mAb could restore cytotoxicity. Thus, it appears that CD94 molecules may function as HLA-class I-specific receptors primarily in NK cells, which do not express another receptor with a more defined specificity for given HLA class I alleles (e.g. p58 or p70).

In this context, it is possible that CD94 molecules represent a more primitive type of receptor for HLA class I molecules which evolved before p58 and p70 receptors; thus CD94 might be able to recognize the products of different class I loci due to the recognition of a conserved epitope (Bw6-related) that is not recognized by the bona fide receptors (i.e. p58 and p70).

Along this line, the CD94 receptor may not represent the human counterpart of the murine Ly49 receptors. Indeed both are type 2 proteins that would appear to recognize MHC class I alleles belonging to different loci.

As previously shown for the HLA-C specific receptors, CD94 can also mediate opposite functional effects. Thus, a major fraction of $CD94^+$ clones is triggered, rather than inhibited, by anti-CD94 mAb in a redirected killing assay (104). Activating but not inhibiting forms of CD94 molecules could induce Ca^{++} mobilization upon mAb-mediated cross-linking. Preliminary biochemical analysis is suggestive of differences in molecular weight between inhibiting and activating CD94 molecules. Remarkably, CD94 molecules appear to be structurally unrelated to both p58 and the HLA-Bw4-specific p70 receptor (see below). Thus, CD94 is surface expressed as a homodimer of 80 kDa which, under reducing conditions, displays a molecular weight of ≈ 43 kDa (101). Recent cloning of the CD94-encoding genes (105) revealed a type II membrane protein encoded by a unique gene of the C-type lectin superfamily. The gene was found on chromosome 12, the syntenic of mouse chromosome 6, where genes of the NK complex (Ly49, NKR-P1) are located. Comparison of the sequences of CD94 and other genes belonging to the human NK complex, including NKR-P1 and NKG2 (106), revealed a homology limited to the structural motifs conserved in the carbohydrate recognition domain. Thus, CD94 appears the only human NK molecule involved in MHC recognition that is structurally similar to the murine Ly49. Although a human homologue of Ly49 has not been identified so far (nor has the murine homologue of p58), these data would suggest that NK receptors serving the same function may belong to two unrelated molecular families. This apparent redundancy of NK receptors for class I molecules may actually be important for sensing different epitopes of the same target molecules (e.g. carbohydrates versus MHC-bound peptides, see above).

The receptor that appears to recognize HLA-B alleles expressing the Bw4 supertypic specificity has recently been identified as a 70-kDa molecule (p70) recognized by mAbs DX9 (68) or Z27 (71). This receptor is selectively expressed only in NK cells recognizing Bw4 and thus differs from CD94. Analysis of the clonal distribution of p58 homologous sequences revealed that $p70^+$ clones consistently expressed transcripts encoding p58-related transmembrane glycoproteins characterized by an extracellular region with three Ig-like domains

(99). Similar findings were obtained by other authors who could identify these transcripts in Bw4-specific NK clones (107).

Similar to the previously defined group 0 NK clones that coexpress both types of HLA-C specific receptors (89), some clones coexpressed inhibiting receptors for the two groups of HLA-B alleles belonging to the Bw4 and the Bw6 supertypic specificities (67). These receptors functioned independently, and since normal cells expressed one or another group of HLA-B alleles, they failed to lyse all allogeneic target cells tested.

3.7 *Coexpression of NK Receptors for HLA-C and HLA-B Alleles*

In addition to NK cells coexpressing both types of HLA-C or HLA-B-specific receptors, some NK clones coexpress inhibiting receptors for both HLA-C and HLA-B alleles (67, 71, 72, 108). Also in these clones the NK receptors function independently, and the mAb–mediated masking of both receptors is required to restore cytolytic activity against target cells expressing both the protective HLA class I alleles (67, 71). It is noteworthy that the p58.1 and the p58.2 receptors (as well as the p70 receptors) are expressed in all individuals regardless of their HLA class I haplotype. This is in line with the concept that class I allele-specific receptors can be expressed regardless of their ability to recognize self–class I alleles (87, 89). However, on the basis of the experimental evidence that self-MHC class I molecules are responsible for shaping the NK receptor repertoire (39–43), these inhibiting receptors for non-self-alleles must necessarily be coexpressed in individual NK cells with inhibiting receptors for self-alleles in order to prevent cytolytic activity against autologous cells.

The simultaneous expression on NK clones of inhibiting receptors for different HLA-class I alleles has provided the molecular basis for explaining the cytolytic pattern of previously defined groups of alloreactive NK cell clones. Thus, while the isolated expression of either p58.1 or p58.2 receptors correlated with group 1 or group 2 NK clones, the expression of p70 alone determined the group 3 specificity, and the combined expression of p70 and p58.1 dictated the specificity of group 5 NK clones (71).

3.8 *Shaping the NK-R Repertoire During Development: Regulation by Positive Selection?*

As discussed above, all NK cells express at least one type of inhibiting receptor for at least one self-HLA class I allele. This implies that, to avoid autoimmune phenomena, the NK cell system has evolved a mechanism of developmental control possibly based on positive selection of NK cell progenitors expressing inhibiting receptors for self–class I alleles. If such interaction takes place, the NK cell will be allowed to undergo further maturation. In contrast, NK cells that

Table 1 The human NK receptors for HLA-class I antigens

Reactive mAbs	Putative ligands	Function	ARAM-like motif*	cDNA	Protein structure	Molecular mass (kDa)	Protein denomination
EB6/XA141	HLA-C "group 1"	inhibitory	+	EB6-cl.42	2 Ig domains	58	p58.1
EB6/XA141	HLA-C "group 1"	inhibitory	+	EB6-cl.47.11	2 Ig domains	58	p58.1
EB6/XA141	HLA-C "group 1"	activatory	–	EB6-act I	2 Ig domains	50	p50.1
GL183/y249	HLA-C "group 2"	inhibitory	+	183-cl.6	2 Ig domains	58	p58.2
GL183/y249	HLA-C "group 2"	inhibitory	+	183-cl.43	2 Ig domains	58	p58.2
GL183/y249	HLA-C "group 2"	activatory	–	183-act I	2 Ig domains	50	p50.2
—	?	?	–	cl.39	2 Ig domains	?	—
DX9/Z27	HLA-Bw4	inhibitory	+	cl.11/AMB11	3 Ig domains	70	p70
DX9/Z27	HLA-Bw4	inhibitory	+	cl.2/NKB1	3 Ig domains	70	NKB1/p70
—	?	?	+	cl.5	3 Ig domains	?	—
3B1/XA185	Various HLA-class I	?**	–	CD94-LL288	C-type, lectin (type II)	43	Kp43/CD94

*Unlike the conventional ARAM motif characterized by two YXXL sequences spaced by 6–8 amino acids, in this case the spacing is 26 amino acids.
**Although both inhibitory and activatory forms of Kp43/CD94 molecules have been described, it is not clear to which of these forms the cDNA CD94-LL288 corresponds.

do not express self–class I–specific receptors would fail to further differentiate. As mentioned previously, frequently NK cells coexpress inhibiting receptors for non-self-MHC class I alleles. Although expression of these receptors is clearly not useful, it is equally harmless to the host. These data suggest that the expression of inhibiting receptors on NK cell subsets during development may reflect a stochastic process in which the various NK-R-encoding genes are regulated in an independent fashion. The hypothesis that the NK-R expression represents the result of a stochastic event is in agreement with the finding that NK cells may express one or more receptors for self-alleles as well as additional receptors for nonself. Conceivably, the fact that NK cells may express only one type of inhibiting receptor for a given self-allele confers an advantage to the host. Thus, lack of expression or peptide-induced alteration of a single MHC–class I allele would be sufficient for NK cells to detect and kill the abnormal cells. It is evident that this control system has major advantages over a system in which single NK cells express receptors for all self-alleles, or, alternatively, a single type of receptor specific for monomorphic determinants of MHC class I molecules.

3.9 *HLA-Class I–Specific Receptors of NK Type on a Subset of Human CTLs*

Resting T lymphocytes do not display spontaneous cytolytic activity against NK-susceptible target cells; however, upon cell activation and culture in IL-2, a fraction of CTLs expressing either TCR α/β or γ/δ has been shown to display NK-like activity. For example, in early studies of T cell cloning, we showed that approximately 1/3 $CD8^+$ CTLs could lyse K562 target cells (109). Thus, upon activation, CTLs characterized by this functional property could be potentially harmful to self-cells, unless they display a safety device preventing autoaggression.

When p58 molecules were first identified in NK cells, a small but sizeable fraction of peripheral $CD3^+$ cells was found to express p58 molecules as well (86, 87). This finding and a subsequent report by Ferrini et al (110) suggested a possible role for class I–specific receptors of NK type in preventing lysis of autologous cells. On the basis of this hypothesis, Mingari et al (111) analyzed whether antibody-mediated masking of class I molecules on resistant target cells could induce their lysis by CTL clones active against K562 target cells. All CTL clones with this function were found to lyse both allogeneic and autologous target cells in the presence of anti-class I mAbs. These data suggested that inhibiting interactions involving class I molecules on target cells and specific NK-type receptors on T cells may represent a general mechanism operating in this subset of CTLs to prevent lysis of autologous cells.

Analysis of T cell clones with the panel of available anti-NK-R mAbs revealed the existence not only of the HLA-C specific p58 receptors, but also of HLA-B specific receptors including CD94 (111) and the p70 (68). Of major importance was the finding that these receptors inhibited not only the spontaneous but also the TCR–mediated target cell lysis or lymphokine production (110, 111). Similar data have been obtained by Phillips et al (112), who showed that CTL clones may express HLA class I–specific receptors that inhibit the superantigen-induced cytotoxicity against target cells that have been transfected with HLA-A, B, or C molecules.

While the receptors for Bw4 have been directly identified with the NKB1/p70 molecule, the presence of the receptors for other HLA alleles has been inferred by the protective effect on target cells exerted by the expression of these alleles. These data are in agreement with our observations that at least four distinct class I–specific NK receptors can be revealed on T cells by the use of specific mAbs. The expression of HLA class I–specific inhibiting receptors on a subset of human CTLs poses important questions as to the ability of these receptors to work against the main function of CTLs, which is to lyse cells that present foreign peptides. It has been speculated that such inhibiting receptors may play a general role in the regulation of CTL responses, perhaps preventing autoimmunity against normal tissues when T cells are activated in the course of inflammatory responses (112). Although these ideas may well be true, it is important to stress that only a fraction of CTLs express these receptors. Thus, the safety device provided by the class I–specific inhibiting receptors may apply to only a (minor) subset of CTLs, which perhaps represent a distinct set of T cells. This concept is supported by preliminary data showing that CD28, the key costimulatory molecule for T cell activation, is not expressed in CTL clones expressing NK receptors for MHC class I. It will be important to further define the functional and phenotypic properties of these cells and to analyze whether they express a wide or a restricted TCR repertoire.

Regardless of the above considerations, it is evident that the analysis of the inhibiting signal in T cells (for which the activation pathways are much better understood than in NK cells) will clarify the activation pathway along which the NK receptor-mediated inhibition occurs as well as which molecular mechanisms are involved.

4. NON–MHC SPECIFIC NK RECEPTORS

A number of surface molecules have been involved in the process of NK cell activation or inhibition. The ability of NK cells to efficiently lyse MHC-negative target cells implies the existence of activating receptor(s) recognizing non MHC-encoded ligands. Among these, the NKR-P1, a disulphide-linked

homodimer expressed on all rodent NK cells (113–115), might play a relevant role. NKR-P1 is an activating receptor mediating natural killing upon binding carbohydrate ligands on NK-sensitive targets. The production of a soluble form of rat NKR-P1 (116) showed that it binds to NK-sensitive target cells but not to NK-resistant cells. In addition, the sNKR-P1 blocked the killing of NK-sensitive targets by freshly isolated polyclonal NK cells. On the other hand, anti-NKR-P1 antibodies did not block cytotoxicity mediated by NK cells. This may be due to the expression of more than one NKR-P1 family member on NK cells or to the fact that monoclonal antibodies to NKR-P1 do not bind to an appropriate epitope. Thus molecules belonging to the NKR-P1 family may be responsible for triggering NK cells when no interaction between protective alleles and receptors delivering negative signals occurs. The affinity of the interaction between NKR-P1 and carbohydrates might dictate the activation or the inactivation of the triggering event. A human homologue of mouse NKR-P1 has recently been characterized and cloned (117). Comparison of the predicted amino acid sequences of human, mouse, and rat NKR-P1A indicated 46% homology. The genes for NKR-P1A are on human chromosome 12 (syntenic of mouse chromosome 6). The NKR-P1A molecule is expressed on a subset of human NK cells (whereas it is expressed on all rat NK cells) and on a subset of T cells including both $CD4^+$ and $CD8^+$ T cells. Anti-NKR-P1 mAbs (DX12) did not affect the lysis of K562 target cells but, at least in some instances, blocked the lysis of P815 target cells in redirected killing assays (117).

Another member of the family of receptors encoded by the NK complex is the CD69 antigen (118, 119). The CD69 molecule is expressed by hematopoietic cells soon after activation (120). Anti-CD69 mAbs can stimulate lymphocyte proliferation (121) and NK cell–mediated cytotoxicity in redirected killing assays (122). The NK activation induced by anti-CD69 mAbs is inhibited by the simultaneous mAb-induced cross-linking of MHC-specific inhibiting receptors thus indicating that, similar to all the other known activating receptors, the CD69-dependent pathway of NK cell activation is under the control of MHC-specific inhibiting receptors (122).

Additional surface molecules involved in NK cell activation/inhibition have been proposed in recent years; these include CD16 (123), CD2 (124), a P38 molecule recognized by the Cl.7 mAb (125), a p70 molecule recognized by the PP35 mAb (126), and a p40 molecule recognized by mAbs NKTA 255 and 1F1 (127). With the exception of CD16 and CD2, the precise role of all these molecules in the complex mechanisms controlling NK cell functions remains largely unknown, although it is unlikely that they may recognize MHC class I molecules.

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THE NF-κB AND IκB PROTEINS: New Discoveries and Insights

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KEY WORDS: NF-κB/Rel transcription factors, IκB, inflammatory cytokines, B and T cell activation, signal transduction

ABSTRACT

The transcription factor NF-κB has attracted widespread attention among researchers in many fields based on the following: its unusual and rapid regulation, the wide range of genes that it controls, its central role in immunological processes, the complexity of its subunits, and its apparent involvement in several diseases. A primary level of control for NF-κB is through interactions with an inhibitor protein called IκB. Recent evidence confirms the existence of multiple forms of IκB that appear to regulate NF-κB by distinct mechanisms. NF-κB can be activated by exposure of cells to LPS or inflammatory cytokines such as TNF or IL-1, viral infection or expression of certain viral gene products, UV irradiation, B or T cell activation, and by other physiological and nonphysiological stimuli. Activation of NF-κB to move into the nucleus is controlled by the targeted phosphorylation and subsequent degradation of IκB. Exciting new research has elaborated several important and unexpected findings that explain mechanisms involved in the activation of NF-κB. In the nucleus, NF-κB dimers bind to target DNA elements and activate transcription of genes encoding proteins involved with immune or inflammation responses and with cell growth control. Recent data provide evidence that NF-κB is constitutively active in several cell types, potentially playing unexpected roles in regulation of gene expression. In addition to advances in describing the mechanisms of NF-κB activation, excitement in NF-κB research has been generated by the first report of a crystal structure for one form of NF-κB, the first gene knockout studies for different forms of NF-κB

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and of IκB, and the implications for therapies of diseases thought to involve the inappropriate activation of NF-κB.

1. INTRODUCTION

Ten years ago Sen & Baltimore (1) first described NF-κB as a B cell nuclear factor that bound a site in the immunoglobulin κ enhancer. In the same year, these researchers also showed (2) that NF-κB could be activated in other cells by exposure to stimuli such as phorbol esters, and that this activation was independent of protein synthesis. In the next few years, functional NF-κB binding sites were found in the promoters of many genes, most of which were not B cell specific. These promoter/enhancers included IL-2, IL-6, GM-CSF, ICAM-1, and class I MHC. Typically, NF-κB binding sites serve as inducible transcriptional regulatory elements that respond to immunological stimuli such as TNF, IL-1, LPS, or T cell activators. However, the range of inducers is not limited to these mediators of immune function; other stimuli such as UV irradation, growth factors, and viral infection also activate NF-κB. The basis for the latent nature of NF-κB and for its inducibility is the association of NF-κB with a cytoplasmic inhibitory protein called IκB (3). The release from IκB allows for the extraordinarily rapid appearance of NF-κB in the nucleus. Thus, certain genes regulated by NF-κB can be transcriptionally activated within minutes following exposure to the relevant inducer.

Ten years after its discovery, the NF-κB and IκB field remains a lively arena for research. Five members of the mammalian NF-κB/Rel proteins have been identified that are characterized by the Rel homology domain (RHD), an N-terminal region of approximately 300 amino acids. These proteins are members of an evolutionarily conserved family of proteins, some of which regulate body pattern formation and immune function in insects. Consistent with a complex system for regulatory control, there are multiple forms of IκB proteins characterized by several copies of the so-called ankyrin repeat. Recent research has elaborated several of the critical aspects of signaling that mediate NF-κB translocation in response to inducer. Gene knockout studies firmly establish a role for NF-κB in immune function and eliminate any models that claim simple redundancy for the functions of the different NF-κB/Rel proteins. Regulation of NF-κB by the network of regulatory cytokines and other immune function modulators is now well established and is growing in complexity. Additionally, the gene deletion studies as well as other approaches provide support for a role of the NF-κB proteins in functions beyond immunity and inflammatory responses, including roles in liver development and in several disease processes.

Two recent *Annual Reviews* chapters (4, 5) have covered NF-κB in great detail through much of 1993. These and other reviews (6–12) offer a thorough background on NF-κB. The aim of this review is to provide coverage of some of the significant advances in NF-κB over the last two years. Many reports involving NF-κB have appeared during this time; due to space limitations, it is impossible for this review to cover all of these important results. In addition, reviews reference previous work where appropriate.

2. THE NF-κB/REL AND IκB PROTEINS

Presently, five members of the mammalian NF-κB/Rel family have been cloned and characterized. These are c-Rel, NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), and RelB (4–12). The Rel homology domain (RHD; see Figure 1) found in each of these proteins functions in DNA binding, dimerization, and interactions with IκB forms. Two of the proteins, NF-κB1 (p105) and NF-κB2 (p100), contain multiple copies of the so-called ankyrin repeat at their C-termini.

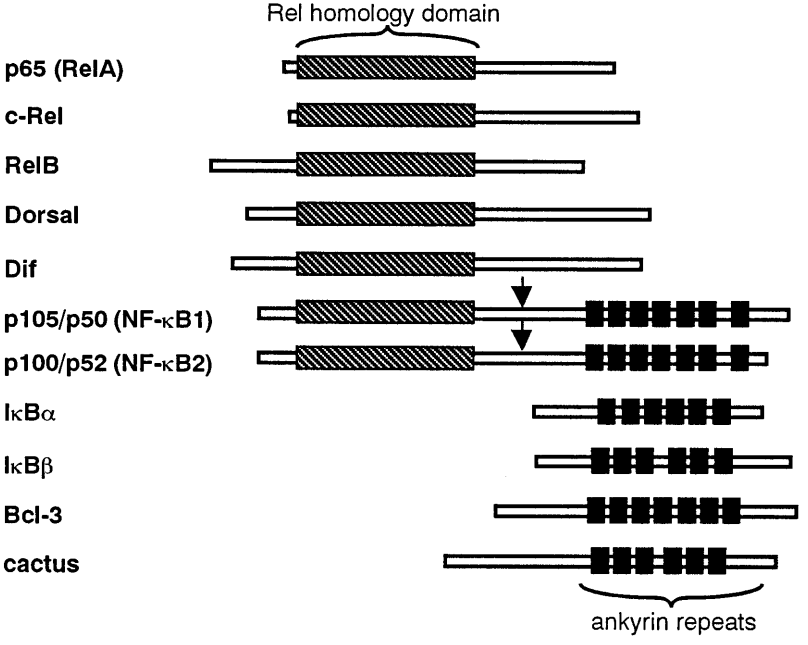

Figure 1 The NF-κB/Rel and IκB families of proteins. The NF-κB/Rel family is characterized by the presence of the Rel homology domain. The IκB proteins have multiple copies of the ankyrin repeat. NF-κB1 and NF-κB2 are proteins that contain both the Rel homology domain and ankyrin repeats. Dorsal, Dif, and Cactus are Drosophila proteins. See text for discussion.

Processing of these proteins (see below) leads to the production of the p50 and p52 subunits. The p100 and p105 proteins serve regulatory functions in the cell (see below) and should not be considered exclusively as precursor forms. Furthermore, some evidence suggests that alternative splicing of the p105 mRNA leads to the translation of additional forms of this protein (13).

2.1 *NF-κB Is a Dimer of Variable Subunits*

Active, DNA-binding NF-κB is a dimer. Classic NF-κB (the dimer of p50 and RelA) has been the most intensively studied, although many other homo- and heterodimers have been described. Certain dimers apparently do not exist; for example, RelB dimerizes only with p50 or p52 (14). Each of the various NF-κB dimers may exhibit distinct properties. For example, binding site preference has been identified for certain dimers. Classic NF-κB binds the sequence 5′ GGGRNNYYCC 3′, whereas the RelA/c-Rel dimer binds to a sequence (5′ HGGARNYYCC 3″; H indicates A, C or T; R is purine; Y is pyrimdine) (15). Selective binding sites for RelA/c-Rel heterodimers are found in the promoters of several inducible genes, including those encoding tissue factor and GM-CSF (15); the RelA/c-Rel dimer is inducible by LPS or by cytokines. The ability of different dimers to recognize slightly different DNA targets increases the ability of NF-κB subunits to differentially regulate gene expression. More examples of biologically relevant targets for the different dimers and of possible roles in gene-specific transcription are needed. Additional differences between the NF-κB dimers include cell type specificity, differential subcellular localization, differential interactions with forms of IκB, and differential activation (4, 5).

2.2 *Dimers of NF-κB Are Targeted by Monomers of IκB*

Initial characterization of cytoplasmic NF-κB revealed that it was associated with either of two forms of IκB–IκBα or IκBβ (4, 5). The cloning of IκBα (16, 17) allowed progress to be made at several levels. First, it was established that IκBα contains ankyrin repeats (Figure 1). Second, IκBα retains NF-κB in the cytoplasm through masking of the nuclear localization sequences (4, 5, 9). Third, the identification of IκB as an ankyrin repeat containing protein allowed for the prediction that the function of the homologous ankyrin repeats in the precursors of the p50 subunit (NF-κB1) and of the p52 subunit (NF-κB2) was as an intramolecular IκB (see 4, 5). Thus precursors dimerize with another member of the Rel family through the RHD, and the C-terminal ankyrin repeat region functions to retain this dimer in the cytoplasm (also see discussion below). Finally, IκBα exhibited homology with a protein encoded by the gene *bcl-3* found to be translocated in certain lymphomas (see 4, 5, and below).

Immunoprecipitation studies indicate that IκBα is associated predominantly with c-Rel- and RelA-containing dimers. Studies indicate that certain non-Rel

or RelA-containing dimers are likely not to be strongly regulated by IκB. For example, IκBα exhibits a lower affinity for RelB-p52 heterodimers than for RelB-p50 dimers (18), and this likely contributes to constitutive nuclear levels of the former factor. RelB complexes in lymphoid cells may have a lower affinity for IκBα due either to a modification to RelB or to a cell-specific cofactor (19). IκB interaction with NF-κB subunits occurs with residues in the Rel homology domain, and presumed contacts in or around the nuclear localization sequence (NLS) appear to play critical functional roles in inhibiting nuclear localization of NF-κB (4, 9). In addition, data indicate that a single IκB targets the NF-κB dimer (20, 21).

IκBα can be divided into three structural domains: a 70-amino-acid N-terminal region, a 205-amino-acid internal region that is composed of ankyrin repeats, and a C-terminal 42-amino-acid region that contains a so-called PEST region. Mutation and protease sensitivity studies indicate that deletion of the N-terminal or C-terminal region does not inhibit the ability of IκBα to interact with NF-κB (20, 22). However, deletion of the C-terminus does block the ability of IκBα to inhibit DNA binding of NF-κB (20). Mutations within the ankyrin repeat block interactions with NF-κB (4, 5). The discussion below describes important regulatory aspects of the N- and C-terminal regions of IκBα.

Other forms of IκB include the precursors NF-κB1 and NF-κB2, IκBγ (an independent protein derived from a unique transcript from NF-κB1) which appears to be limited only to mouse B cells, and Bcl-3 (Figure 1; see 4, 5). The recently cloned IκBβ is discussed below. Precursor proteins can dimerize with other NF-κB subunits to form dimer molecules that cannot bind to DNA and that cannot translocate into the nucleus (4, 5). NF-κB2 precursor p100 or its C-terminus can form a trimeric complex with a dimer of NF-κB subunits (23, 24), suggesting a different mechanism whereby precursors function in an IκB-like role. Bcl-3 is nuclear in its localization and functions as a transcriptional activator with the p50 or p52 homodimer. Thus the ability of Bcl-3 to interact with these forms of NF-κB results in transcriptional activation rather than an inhibition of nuclear transport or an inhibition of DNA binding (4, 5). Consistent with these results is the observation (25) that the NLS region of p50 is apparently not contacted by Bcl-3, in contrast to the interactions between IκBα and NF-κB subunits.

2.3 *IκBβ Appears To Have Properties Distinct from IκBα*

As stated above, purification of NF-κB revealed that two forms of IκB were associated with NF-κB dimers. One form, IκBα, is described above and has been intensively studied. Recently, the 46-kDa IκBβ was purified to homogeneity, and a cDNA clone was derived (26). Like IκBα, IκBβ contains ankyrin repeats (Figure 1) and is associated with NF-κB forms in the cytoplasm of various

cells. Like IκBα, IκBβ preferentially interacts with dimers that contain c-Rel or RelA. mRNA for IκBβ is widely expressed, with an especially high level in the testis. Where IκBα is targeted by a signaling pathway initiated by TNF, IL-1, LPS, and PMA, IκBβ reportedly is targeted only by pathways initiated by LPS or by IL-1 (at least in the 70Z/3 pre-B cell line and in the Jurkat T cell line). Further discussion of IκBβ follows in the section on signaling and activation of NF-κB.

2.4 *IκB-R and IκBL: Functional IκB Molecules?*

A cDNA clone for an ankyrin repeat-containing protein with homology to mammalian and invertebrate IκB proteins was recently reported (27). This protein, IκB-R, is most similar to the Drosophila IκB homolog Cactus (Figure 1 and see below) and is expressed in epithelial cell lines but not in fibroblasts. Two forms of RNA are detected (the smaller RNA is not large enough to encode the described protein), with the larger form expressed in heart and skeletal muscle but not in brain, lung, liver, or kidney. The IκB-R protein inhibits the DNA binding activity of the p50/RelA dimer and the p50 homodimer but not that of the RelA homodimer, suggesting a preferential interaction with the 50-kDa NF-κB1 subunit. A regulatory role for IκB-R has not yet been identified.

Another cDNA clone encoding a protein (IκBL) with homology to IκB family members has been reported (28). The protein contains two complete and one partial ankyrin repeat and is encoded by a gene in the major histocompatibility complex. No functional studies on this protein have been presented.

3. STRUCTURAL AND EVOLUTIONARY STUDIES ON NF-κB

3.1 *Crystallography of NFκB1 Bound to DNA*

Two groups recently described the crystal structure of the 50-kDa NF-κB1 homodimer bound to a κB site (29, 30). In contrast with the relatively small DNA binding domains of many other transcription factors, the DNA binding domain of NF-κB1 encompasses large regions of the RHD. These studies reveal that the RHD folds into two distinct domains similar to those in the immunoglobulin superfamily. DNA contacts are made on the κB site by both domains, and the C-terminal regions of the RHD constitute the dimerization interface. The overall structure is striking and butterfly-like (see Figure 2). The general relatedness of the RHD in each of the immediate NF-κB family members strongly suggests that a similar overall structure will constitute the DNA binding and dimerizations domains of each family member. However, distinct sequence elements within these domains likely determine different functional properties

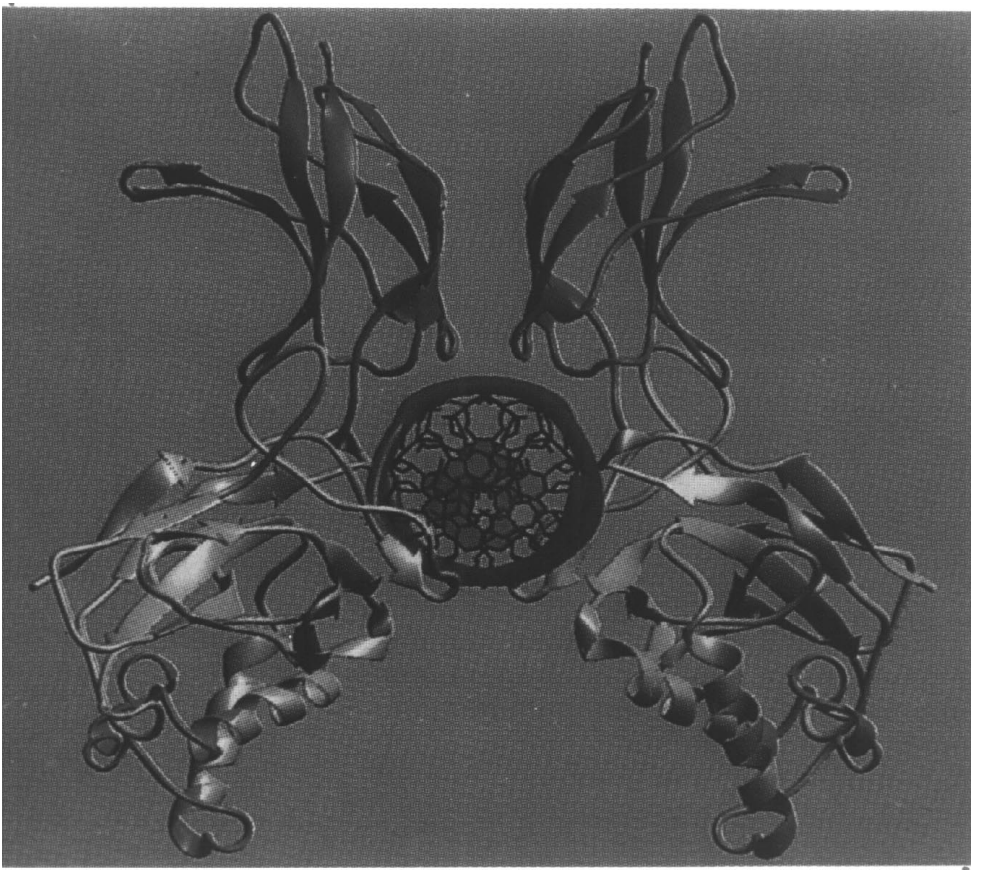

Figure 2 Structure of the Rel homology domain of the p50 NF-κB1 homodimer and its target DNA sequence (see 29, 30). The more N-terminal region of the RHD is shown toward the bottom, and the more C-terminal dimerization region at the top. DNA contacts are made in both domains. (Photograph from Dr. S. Harrison.)

of the different subunits (31). Consistent with previous deletion studies, it is proposed that amino acids in and around the NLS will constitute a composite surface for interaction with IκB. Another important feature is that the minor groove is exposed in the center of the κB site, allowing for the interaction of HMG-I(Y) with certain κB sites [see discussion of HMG-I(Y) below].

3.2 *NF-κB/Rel Proteins Are Members of a Larger Family*

The factor NF-AT is strongly implicated in the inducible transcription of the IL-2 gene and other cytokine genes. Interestingly, many similarities exist between the NF-κB and the NF-AT family of proteins (see 32 and references therein). Both groups are inducible by activation of T cells, utilizing cytoplasmic/nuclear translocation as a control mechanism, and this activation is blocked by the important immunosuppressant cyclosporin A. Additionally, both NF-AT and NF-κB interact with members of the Fos/Jun family of proteins (32). Furthermore, the core sequence of the NF-AT recognition site, GGAAAA, is very similar to an NF-κB half-site. The recent cloning of members of the NF-AT family demonstrates the existence of a region of NF-AT similar to the Rel homology domain of NF-κB (32; 33). Overall these observations indicate the existence of an extended family of Rel-like proteins that serve critical roles in immune function.

An impressive structural and functional homology exists between the mammalian NF-κB/Rel proteins and two proteins, Dorsal and Dif (see Figure 1), from Drosophila. Dorsal is involved in critical developmental pathways determining the dorsal-ventral patterning of the embryo (see 34). Dif serves a protective function, regulating bacteriocidal genes in response to endotoxin (35). These proteins are regulated by an ankyrin repeat containing protein known as Cactus (Figure 1; 34, 36), which functions very similarly to mammalian IκB in controlling nuclear/cytoplasmic localization of Dorsal (37).

4. BIOLOGY OF NF-κB

4.1 *Expression and Nuclear/Cytoplasmic Status of NF-κB Forms*

Although NF-κB is generally considered to be cytoplasmic in most cell types until stimulation with an inducer, growing evidence provides a more complex view. First, many cell types appear to have moderate levels of the p50 (NF-κB1) homodimer. A role for this factor in constitutive-type transcription is unclear, but it may provide low levels of transcriptional activity or it may serve as a transcriptional repressor (4, 5). Since the p50 dimer can be targeted by Bcl-3, it may serve as a DNA-binding anchor for transcriptional activation by this IκB-related protein (5). A protein similar to or identical to c-Rel is

nuclear during the S phase of the cell cycle (38). Evidence has been provided that NF-κB (other than the p50 homodimer) is nuclear in corneal keratinocytes (39) and in vascular smooth muscles cells (40). Following is a discussion of some other cells where constitutive activation of forms of NF-κB may play important functional roles.

4.1.1 B CELLS The one widely accepted example of constitutively active (i.e. nuclear) NF-κB is in B cells. B lymphocytes develop by regulating an ordered expression of immunoglobulin genes, and one role for NF-κB in B cells is in regulating the Igκ gene (7). Pre-B cell lines do not exhibit active NF-κB unless stimulated with cytokines or LPS. However, pre-B cells expanded from Whitlock-Witte bone marrow cultures by treatment with IL-7, and then maintained with a stromal cell feeder layer, exhibited active NF-κB and Igκ transcription (41). Recently several groups have shown that the constitutive form of NF-κB in mature B cell lines is largely the p50-c-Rel heterodimer (42, 43). The p50-RelA heterodimer is found at expected levels in the cytoplasm but is not nuclear in these cells. The mechanism accounting for nuclear levels of c-Rel-p50 is not fully clear but may be explained by significantly increased instability of IκBα and by increased transcription of the c-Rel gene (43, 44).

The functional significance of the constitutive nuclear activation of the c-Rel-p50 heterodimer, and the relative lack of p50-RelA, in mature B cell lines is not clear. Both heterodimers appear to activate Igk gene expression effectively (43). NF-κB RelA is a potent activator of *c-myc* transcription, as compared to c-Rel, and anti-IgM treatment of a B cell line resulted in increased forms of NF-κB in the nucleus, increased transcription of *c-myc*, and apoptosis (44). Thus, activation of the p50-RelA heterodimer may be deleterious to normal B cell function, resulting in upregulation of *c-myc* and apoptosis. Additional evidence for an important role for NF-κB in B cell biology comes from studies on the B cell surface receptor CD40, which is a member of the TNF receptor family. Engagement of the CD40 receptor activates NF-κB in B cell lines (45, 46), leading to the transcription of a gene encoding a zinc finger protein, A20, which inhibits apoptosis (45). CD40 signaling blocks spontaneous apoptosis of B cells in germinal centers and may be important in selecting B cells undergoing somatic mutation (see 46). The studies with anti-IgM utilized WEH1 231 cells, while the studies with CD40 utilized Louckes and BJAB cells. Thus, NF-κB may have both positive and negative effects on apoptosis in B cells. Additionally, activation of Nf-κB by engagement of CD40 is likely to play other important roles in B cells.

4.1.2 THYMOCYTES Experiments utilizing freshly isolated thymocytes found that several factors that bind to a κB site are constitutively activated in these cells

(47). These factors include p50 homodimers, the p50-RelA heterodimer, and c-Rel. Disruption of the thymic microenvironment led to the loss of these binding activities, which suggests that an interaction between lymphocytes and stromal cells provides a stimulus for the activation of NF-κB/Rel nuclear proteins. Some evidence suggests that c-Rel plays a role in the transition from $CD4^+/CD8^+$ double positive cells to $CD4^+$ or $CD8^+$ single positive cells. Gene knockout studies with p50 or c-Rel do not indicate a role for these subunits in T cell development (see below) but do not rule out a role for RelA.

4.1.3 NEURONS Numerous reports analyze the status of κB binding activities and NF-κB-like forms in the brain. A brain κB binding protein, called BETA, has been described (48). The BETA complex can be supershifted with antibodies against a large zinc finger protein called MBP-2/AGIE BP-1 (49). Other work has identified two κB binding complexes, DBF 1 and 2, in the developing rat brain (50). Cross-linking identified the molecular weights of the factors as approximately 110 and 115 kDa. However, these factors are not related to the NF-κB1 and or NF-κB2 factors. Immunohistochemical evidence (51) suggests that both NF-κB p50 and RelA are constitutively active in some neurons of the embryonic rat. This latter work also identified nuclear NF-κB by gel mobility shift assay. Furthermore, κB-dependent gene expression could be detected in neuronal cell cultures. Experimentation has revealed that vascular adhesion molecule-1 (VCAM-1) is expressed in the developing central nervous system on neuroepithelial cells (52), which are precursors of glial cells and neurons. Utilization of P19 embryonic carcinoma cells as a model of neural differentiation revealed that expression of VCAM-1 correlated with induction of nuclear p50-RelA dimers. NF-κB activity in the brain may be involved with expression of HIV in the central nervous system and may participate in normal brain function.

4.2 *Transcriptional Regulation by NF-κB Subunits*

Consistent with their roles as transcription factors, c-Rel, RelB, and RelA contain transcriptional activation domains (53, 54, and see 55). Additionally, both c-Rel and RelA interact with the TATA-binding protein (TBP), and the C-terminus of RelA interacts with the basal factor TFIIB (56 and references therein). Studies in vivo and in vitro indicate that different NF-κB dimers have different transcriptional activation properties (57, 58). Strong evidence indicates that interactions between NF-κB and other transcription factors influence the ability of NF-κB to regulate gene expression in a selective manner. As described below, interactions between NF-κB proteins, HMG-I(Y), IRF-1, and bZIP proteins regulate inducible expression of the interferon β gene. Interactions between NF-κB proteins and bZIP proteins are implicated in the inducible regulation of the genes encoding IL-8, E-selectin, and G-CSF,

for example (see below). A complex that binds to enhancer A in the class I MHC promoter and that is modulated by interferon γ or glucocorticoids reportedly contains the p50 NF-κB subunit in association with the bZIP protein fra-2 (59). Additionally, a DNA-binding complex containing C/EBP and NF-κB proteins has been identified in avian lymphoid cells (60). An interaction between the NF-κB subunit RelA and the zinc finger protein Sp1 has been identified and appears to regulate transcription directed by the HIV-1 LTR (61). The functional outcome of transcriptional induction based on some of these interactions likely involves cooperative DNA binding as well as transcriptional synergy.

4.3 *Genes Regulated by NF-κB*

Extensive research has established a clear role for NF-κB in the inducible regulation of a wide variety of genes involved in immune function and inflammation responses (for example, GM-CSF, IL-6, IL-8, IL-2, IL-2Rα, etc). An extensive list of genes regulated by NF-κB is provided in previous reviews (4, 5). Following is a discussion of several of these.

The group of genes encoding cell adhesion molecules has been studied extensively for an involvement of NF-κB in their regulation. Vascular cell adhesion molecule-1 (VCAM-1) is a cell surface protein typically found on endothelial cells following exposure to TNF, IL-1, or LPS. VCAM-1 is a member of the immunoglobulin superfamily and binds circulating monocytes and lymphocytes expressing $\alpha 4\beta 1$ or $\alpha 4\beta 7$ integrins and likely participates in the recruitment of these cells to sites of tissue injury. VCAM-1 is also implicated in other cellular processes because it is expressed in the developing central nervous system, in human lymph nodes, and on bone marrow stromal cells. The promoter of the VCAM-1 gene contains two essential NF-κB sites (62), which are not sufficient for activation, and may require an IRF-1 site that is located 3′ to the TATA box (63). Interestingly, IRF-1 was found to be inducible by cytokine treatment. Furthermore, IRF-1 physically interacts with NF-κB subunits, and its binding is stimulated by binding of NF-κB and HMG-I(Y). The functional requirement for IRF-1 may explain the cell-type specificity of the VCAM-1 promoter. IRF-1 and NF-κB may be important in the cytokine-induced regulation of multiple promoters. Other genes encoding cell adhesion molecules, such as E-selectin, ICAM-1, and MAd-CAM-1, are also regulated by NF-κB (64–67). Expression of E-selectin requires NF-κB, the bZIP protein ATF-2, and HMG-I(Y) (64, 65). Additionally, interactions between HMG-I(Y), NF-κB, and an ets-like protein (Elf-1) reportedly regulate expression of the IL-2 receptor α gene (68).

Regulation of the interferon β gene has been intensively studied by Maniatis and colleagues (69, 70). The human gene is induced by viral infection of cells or treatment with double-stranded RNA. One of the regulatory sites (GGGAAATTCC; PRDII) in the IFNβ promoter interacts with both NF-κB and the high

mobility group protein HMG I(Y). HMG I(Y) stimulates the binding of NF-κB to PRDII by binding to the A/T-rich core sequence. Mutations that block either NF-κB or HMG I(Y) binding significantly block activation. These workers propose a model in which interactions between bZip proteins (ATF-2 and c-Jun), IRF-1, HMG I(Y), and NF-κB form a higher order complex, which is required for full activation of the IFNβ promoter (70). Thus a precise arrangement of binding sites and a κB site that interacts with HMG-I(Y) are required for the proper transcriptional induction of the IFNβ gene. Strong similarities exist between the inducible regulation of the IFNβ gene and those encoding adhesion molecules (64).

Other genes regulated by NF-κB that encode proteins playing important roles in immune response and inflammation are the genes encoding the peptide transporter TAP1 and the proteasome subunit LMP2 (71) as well as the MHC class II invariant chain gene (72). Additionally, genes encoding tissue factor (15, 73), inducible nitric oxide synthase (iNOS), and various cytokines are regulated by NFκB. iNOS catalyzes the high output of NO, and the iNOS gene is transcriptionally activated in response to LPS and interferon gamma. The inducible activation of NF-κB stimulates the transcription of the iNOS gene (74), leading to an increase in NO production. As discussed below, the production of NO may feed back to inhibit NF-κB activation. NF-κB is strongly implicated in the transcriptional regulation of several cytokine and growth factor genes, including IL-2, IL-6, IL-8, and G-CSF. IL-2 contains an NF-κB site that has been studied by several groups (7). Activators of T cells such as PMA and ionomycin activate NF-κB and its binding to this site. The immunosuppressant cyclosporin A blocks the activation of NF-κB in response to T cell receptor–mediated signals (75). Signaling through CD28, a costimulatory receptor, also leads to the activation and binding of NF-κB/Rel forms to a recently described CD28 response element (CD28RE) in the IL-2 promoter (76). The CD28RE is found upstream of other genes such as IL-3. Furthermore, expression of the IL-8 gene is regulated by NF-κB (77) and uses the CD28 costimulatory pathway in T cells (78). G-CSF is a growth factor for hematopoietic cells produced by mesenchymal and myeloid cells in response to activation by inflammatory stimuli. A cooperative interaction between C/EBPβ (NF-IL6) and the RelA subunit of NF-κB is important in the regulation of G-CSF in response to TNF treatment (79).

Although NF-κB/Rel proteins are strongly implicated in the regulation of genes involved in the immune system and in inflammation, these transcription factors also regulate genes involved in control of cell growth. Two NF-κB sites have been identified in the *c-myc* promoter/enhancer region (80). The induction of *c-myc* gene expression either by IL-1 or by the HTLV-I tax protein functions

through the two NF-κB sites. As discussed previously, classical NF-κB or p65 homodimers appear to be the best activators of *c-myc* as Rel is only a weak activator. NF-κB binding proteins are also implicated in expression of the translocated *c-myc* allele in Burkitt's lymphoma (81) and in the transcriptional regulation of the p53 gene (82).

4.4 *Roles for NF-κB Subunits Revealed in Gene Knockout Studies*

Knockout of most of the genes encoding NF-κB/Rel subunits and two of the IκB forms has been recently accomplished. The studies confirm the importance of NF-κB/Rel proteins in immune function and have revealed several unexpected findings.

4.4.1 NF-κB1 Mice lacking the p50/p105 (NF-κB1) subunits develop normally but exhibit defects in immune responses involving B cells (83). In these mice, the ability of B cells to proliferate in response to LPS is defective, and the production of antibodies is impaired. Total serum Ig was approximately four-fold lower in the knockout mice, and IgE was reduced approximately 40-fold. This suggests that p50 plays an important role in heavy-chain class switching. Interestingly, p50 is reportedly (84) a critical factor for an IL-4-responsive region in the Ig heavy-chain germline ϵ promoter, responsible for switching to IgE. NF-κB1 $-/-$ mice cannot effectively clear the pathogens Listeria or Streptococcus and exhibit increased resistance to infection by EMC virus (83). This resistance was correlated with increased expression of IFNβ, an important antiviral protein. Thus p50 homodimers likely negatively regulate expression of the IFNβ gene.

4.4.2 RELB Like the NF-κB1 knockout mice, the RelB knockout mice do not show a developmental phenotype but do show defects in normal immune function and in hematopoiesis (85, 86). It had previously been speculated that RelB plays an important role in the constitutive type expression of κB-regulated genes in lymphoid cells. RelB is expressed in the spleen, thymus, lymph nodes, and intestine. In the thymus, RelB transcripts are concentrated in the medulla with high levels of RelB in the nucleus of interdigitating dendritic cells. The RelB $-/-$ mice exhibited multifocal and mixed infiltration of inflammatory cells in several tissues. Additionally, the mice exhibited splenomegaly, reduced antigen-presenting dendritic cells in the thymus, myeloid hyperplasia, and impaired cellular immunity. These studies demonstrate an important role for RelB in immune function and in the differentiation of dendritic cells and thymic medullary epithelial cells. Furthermore, the data show that the loss of RelB cannot be complemented by another member of the NF-κB/Rel

family and, therefore, that there is no simple redundancy within the NF-κB/Rel proteins. It will be important to identify the relevant genes regulated by RelB that contribute to these immunological functions.

4.4.3 RELA The RelA (p65) null mice exhibit a dramatic phenotype–embryonic lethality apparently due to widespread apoptosis within the liver (87). Day-14 embryos exhibit a normal liver phenotype, but by day 16 the liver (specificially hepatocytes) has undergone extensive apoptosis. This phenotype is similar to that observed with the c-Jun knockout and suggests that NF-κB and Jun may serve similar functions in development of the liver (see 87). The normal induction of the p50-RelA heterodimer by TNF was lost in the RelA null mice as was the inducibility of NF-κB-regulated genes such as *IκBα* and *GM-CSF*.

4.4.4 c-REL The c-Rel knockout mouse develops normally, and cells from all hemopoietic lineages are normal; however, mature B and T cells are not responsive to certain mitogenic stimuli (88). The c-Rel $-/-$ animals exhibit normal Igκ levels on splenic B cells and normal IL-2Rα chain on thymic T cells; thus, c-Rel is not required for expression of these genes containing κB binding sites. Proliferation of B cells induced by LPS, CD40, or anti-IgM was defective for unknown reasons. A defective T cell–proliferative response induced by engagement of the T cell receptor was observed, and CD28 costimulation did not overcome this defect. Interestingly, proliferation of cells induced by PMA and ionomycin was near normal. The inability of c-Rel $-/-$ T cells to respond to anti-TCR stimuli and CD28 costimulation was correlated with loss of production of IL-2. Indeed, this proliferative block could be overcome by the addition of exogenous IL-2. Thus, it appears that c-Rel is required for the CD28 responsive element to be functional (see discussion above). Additionally, the c-Rel deficient mice displayed deficient immunoglobulin production in unchallenged animals, and T cell–dependent humoral immune responses to antigenic challenge were also impaired.

4.4.5 IκBα AND BCL-3 IκBα null mice are apparently normal at birth but then enter a wasting phase and die approximately 7 days after birth (89). The runted mice have small spleens and thymuses, enhanced granulopoiesis, and scaly skin. NF-κB is constitutively activated in splenocytes and thymocytes as well as in other cells. Upregulation of some genes expected to be regulated by NF-κB (G-CSF and VCAM-1) is detected. The loss of IκBα leads to the nuclear localization of NF-κB in most cell types, most prominently in the spleen and thymus. This latter result suggests that IκBα is the dominant IκB in these organs. Treatment of IκBα $-/-$ embryonic fibroblasts with TNF leads to activation of NF-κB through targeted degradation of IκBβ, showing that at least

in some cell types this inhibitor can be targeted by TNF. Furthermore, nuclear NF-κB levels remained elevated longer in −/− animals following treatment with TNF, demonstrating a role for IκBα in controlling the postinduction repression of NF-κB. Clearly, the IκBβ knockout studies will be informative in understanding potential differential roles for the two major IκB forms. Evidence for a role in constitutive activation of NF-κB in the phenotype of the IκBα −/− mice is the observation that a cross between these mice and the p50 −/− mice extends the life expectancy of the former (89).

The Bcl-3 gene knockout has been derived very recently. So far, no obvious developmental or immunological abnormalities have appeared in the mice (A Berns, I Verma, personal communication).

5. MECHANISMS FOR THE ACTIVATION AND CONTROL OF NF-κB ACTIVITY

Enormous progress has been made toward identifying the mechanisms whereby NF-κB is activated to move into the nucleus in response to numerous stimuli. Specific molecular events involved in this activation are now well described and offer insights into a fascinating control mechanism. Despite this progress, many important questions remain regarding the pathways and mechanisms of NF-κB activation.

5.1 *Phosphorylation of IκBα Is Coupled To Its Proteolysis via the Ubiquitin Pathway*

In vivo analysis has shown that inducer-mediated activation of NF-κB is correlated with the hyperphosphorylation of IκBα and its subsequent degradation (reviewed in 4–12). Paralleling the loss of IκBα in the cytoplasm is the appearance of NF-κB in the nucleus. Reagents such as antioxidants or certain protease inhibitors that inhibit phosphorylation block the appearance of nuclear NF-κB. Based on studies showing that IκBα is intrinsically unstable when not associated with NF-κB, phosphorylation of IκBα was generally thought to lead to its dissociation from NF-κB, which resulted in proteolysis (9). Recent experiments have offered a different interpretation.

One set of experiments (90–96) showed that hyperphosphorylated IκBα could be coimmunoprecipitated with NF-κB, suggesting that inducible phosphorylation does not cause the dissociation of IκBα from NF-κB. Second, experiments with a class of protease inhibitors called peptide aldehydes demonstrated that these compounds block the degradation of IκBα and the nuclear appearance of NF-κB, but do not block inducible phosphorylation of IκBα (90–97). In addition, these studies led to the important proposal that a target of peptide aldehydes, the proteasome, is responsible for the signal-mediated

degradation of IκBα (97). Like IκBα, IκBβ is targeted for degradation in response to the inducers LPS and IL-1. Evidence that IκBβ is degraded by the proteasome is provided by data that show that peptide aldehydes specific for the proteasome block its degradation (T Finco, J Reuther, A Beg, D Ballard, S Shenolikar, A Baldwin, submitted).

What mechanism targets IκBα, and presumably IκBβ, for degradation? Evidence that phosphorylation precedes degradation (95) suggests that phosphorylation of IκBα is required for degradation. Mutation of two serines near the N-terminus of IκBα, ser32 and ser36, blocked inducible phosphorylation and also blocked degradation (98–100). Phosphopeptide mapping demonstrates that these two serines are inducibly phosphorylated in response to various activators of NF-κB (J DiDonato, M Karin, personal communication). Recent work indicates that IκBα is ubiquitinated in response to inducer, and mutation of ser32 and ser36 inhibits the inducible ubiquitination (101). Importantly, ubiquitinated IκBα is degraded by purified preparations of proteasomes. Mutation of two lysine residues in the N-terminus block ubiquitination and degradation of IκBα but not induced phosphorylation (102). Presumably these two lysines are the targets for uibiquitination. Thus the current model is that targeted phosphorylation of IκB leads to the ubiquitination of this protein, which leads to degradation by the proteasome (see Figure 3). A question raised by these data is, what makes the phosphorylated form of IκB a substrate for the ubiquitination pathway? Possibly the lysine residues to be ubiquitinated are masked by secondary or tertiary structure until the nearby serines are phosphorylated.

Although inducible phosphorylation of IκBα (and presumably IκBβ) plays a critical role in the activation of NF-κB, IκBα is also constitutively phosphorylated. Recent evidence suggests that casein kinase II (CKII) can associate with and phosphorylate the C-terminal region of IκBα (103; J Hiscott, personal communication). Interestingly, deletion of the C-terminus inhibits degradation (but not association with NF-κB) (98, 104, 105) and also abrogates the ability of IκBα to inhibit DNA binding by NF-κB (20). Thus, constitutive phosphorylation of IκBα in the C-terminal region may be required for degradation, possibly mediated by the PEST residues found in this domain. Additional roles for constitutive phosphorylation of IκBα may include regulation of the stability of free IκB or assembly with NF-κB subunits.

5.2 *NF-κB Subunits Are Targeted for Inducible Phosphorylation*

Both the NF-κB1 (p105) and NF-κB2 (p100) proteins are cytoplasmic and are associated with different NF-κB/Rel proteins. As discussed above, this association may be as dimers or, as recently proposed, trimers. Several of the NF-κB

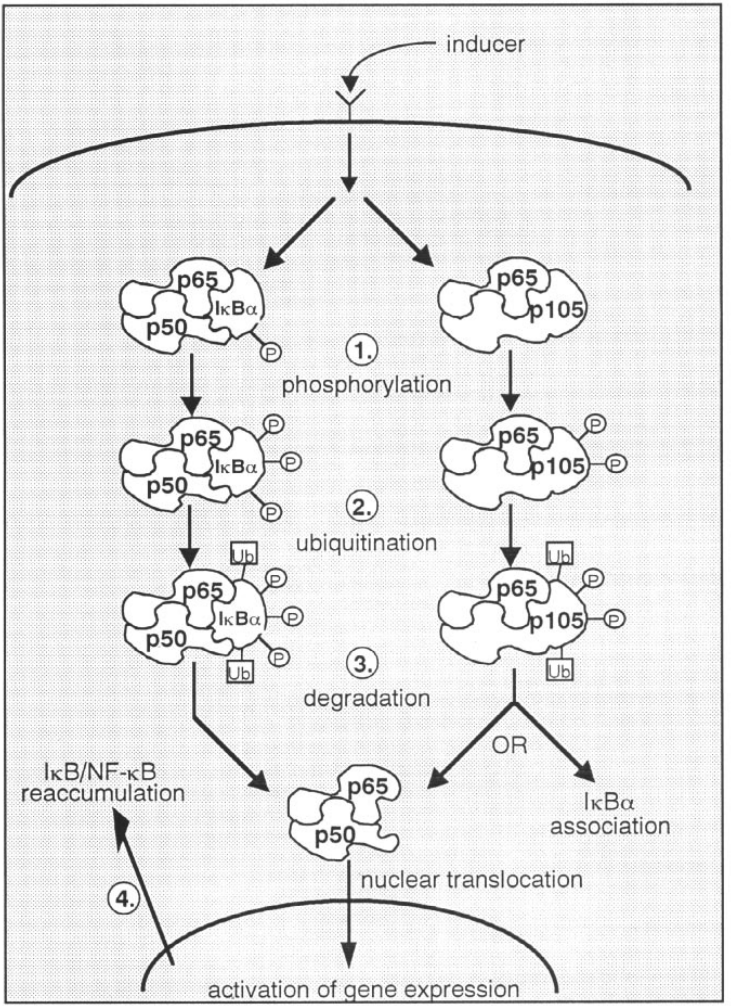

Figure 3 Generalized NF-κB activation scheme. Following exposure of a cell to inducer, IκB (either IκBα, IκBβ or one of the precursor proteins) becomes phosphorylated (*step 1*) by a presently unknown kinase(s). (Note that IκBα exhibits a basal level of phosphorylation.) The IκB form then becomes ubiquitinated (*step 2*) and degraded by the proteasome (*step 3*). In the case of p105, the degradation is partial (generating p50). NF-κB then translocates to the nucleus where it activates a variety of genes (*step 4*) including IκBα and p50/p105. See text for details.

inducers lead to the degradation of the p105 precursor protein with the appearance of the p50 form (4, 5). This degradation is limited to the C-terminal half of the protein (97). As with IκBα targeting, degradation appears to be preceded by inducible phosphorylation (106, 107) and is mediated by ubiquitination and processing by the proteasome (see Figure 3; 97). Degradative processing of p105 that is in association with other NF-κB subunits (for example, RelA) may lead to nuclear translocation directly or to association with an IκB form (see Figure 3).

Reagents that induce NF-κB activation via the inducible phosphorylation of IκB reportedly also lead to the inducible phosphorylation of subunits other than those for the precursor/IκB-like forms. For example, the RelA subunit becomes rapidly phosphorylated in response to TNF (108; D Wang, T Finco, A Baldwin, unpublished). A role for this inducible phosphorylation has been suggested to involve enhancement of DNA binding (106) but may also be correlated with release from IκB, nuclear translocation, activation of transcription functions, or for differential targeting of NF-κB forms that may be associated with IκBα or IκBβ (see below).

5.3 *Mutual Regulation of NF-κB and IκB*

Initial studies that demonstrated the correlation of IκBα loss with the appearance of nuclear NF-κB also indicated that IκBα levels recovered within an hour or so (see 4, 5 for reviews). This reappearance of IκBα depended on protein synthesis and correlated with the inducible expression of IκBα mRNA. Furthermore, transfection into cells of an expression vector for RelA led to an increase in IκBα mRNA. It was therefore proposed that nuclear NF-κB caused the transcriptional activation of the IκBα gene. Promoter analysis has confirmed that the IκBα gene contains multiple NF-κB binding sites and that these sites are functional in the upregulation of gene expression in response to inducers that activate NF-κB (see 4, 5). Evidence has been presented, however, that adherent monocytes (from which IκBα was initially cloned) upregulate IκBα mRNA through a nontranscriptional event (109). These results indicate that NF-κB and IκB are components of a mutual regulatory system in which the levels of one regulatory component control the activity or quantity of the other.

The rapid reaccumulation of IκBα following its loss is apparently important in reestablishing cytoplasmic pools of NF-κB/IκB complexes. Additionally, this reaccumulation appears to repress NF-κB activity following induction because resynthesized IκBα enters the nucleus, interacts with NF-κB forms, and inhibits DNA binding (110). Consistent with this model is the observation that IκBα $-/-$ cells exhibit high nuclear levels of NF-κB for long times following induction with TNFα (89). A mechanism for removal of NF-κB from the

nucleus is suggested by the presence of a potential nuclear export sequence in IκBα (111). Thus IκBα, and not IκBβ (see below), is considered to be important for the postinduction repression of NF-κB.

5.4 *Similarities and Differences Between IκBα- and IκBβ-Controlled Pathways*

As discussed previously, many similarities exist between IκBα and IκBβ. Evidence indicates, however, that IκBβ does not respond to treatment with TNF or PMA but does respond to LPS or IL-1 in pre-B or Jurkat T cells (26). In addition, IκBβ reportedly does not reaccumulate following induction of NF-κB; thus a persistent activation of NF-κB follows. Mechanisms that would prevent IκBα, which rapidly reaccumulates following induction, from inhibiting the IκBβ-released NF-κB have been proposed. One idea is that NF-κB that is associated with IκBβ would be modified (possibly by phosphorylation) so that it could not be targeted for inhibition by IκBα. Many issues remain to be clarified regarding differences between IκBα and IκBβ. One question is whether different kinases function to target the two forms of IκB. Evidence for this is that IκBβ is targeted for degradation with slower kinetics than IκBα and that there is apparent inducer specificity in the targeting of the two IκB forms. However, recent evidence (J DiDonato, M Karin, personal communication) indicates that serines in the N-terminus of IκBβ, in homologous positions to ser32 and ser36 in IκBα, play critical roles in the targeted degradation of this protein. This would indicate that a kinase that is identical or very similar to the IκBα kinase would target IκBβ. Additionally, it is not clear whether targeting of IκBβ occurs in all cell types (see discussion above relevant to the IκBα gene knockout).

5.5 *Tangled Evidence for the Signal Transduction Pathways*

5.5.1 KINASES AND PHOSPHATASES Much research is now directed at elucidating the signal transduction pathways involved in controlling activation of NF-κB. It is clear that the many different inducers initiate their pathways through distinct receptors. How these different responses converge on IκB is still unknown. Mutations of ser 32 and 36 inhibit activation of NF-κB controlled by T cell activation signals PMA, TNF, LPS, HTLV-I tax, and okadaic acid. Thus, a single kinase activated by multiple pathways may target these residues in NF-κB.

Nevertheless, several kinases have been implicated in the activation of NF-κB. The best data exist for the double-stranded RNA activated kinase (PKR), which phosphorylates IκBα in vitro (112). Furthermore, in vivo inactivation of this kinase inhibits the ability of double-stranded RNA to activate NF-κB (113). However, this inactivation did not block activation of NF-κB by TNF

and presumably other inducers. It would be interesting to map the potential phosphorylation sites on IκBα targeted by dsRNA-activated kinase. Another kinase, Raf-1, has been proposed, to target IκBα (114). Evidence for an involvement of Raf in the activation of gene expression directed through a κB site has been presented through the utilization of constitutively active and dominant negative vectors (115). Activation of Ras or Raf may not lead to the nuclear translocation of NF-κB, but it can activate gene expression through a κB site. The mechanism is unclear but may involve the stimulation of the transcriptional activation domains of constitutively nuclear forms of NF-κB (T Finco, A Baldwin, unpublished). Dominant negative experiments as well as direct expression experiments have implicated PKCζ as a regulator of NF-κB activation (116). Due to their activation by inducers of NF-κB such as inflammatory cytokines, UV, and LPS, there has been speculation that members of the stress-activated protein kinases (SAPKs) or their regulators may play a role in IκBα phosphorylation, but no published data exist regarding this possibility. Additionally, a kinase reportedly associated with NF-κB/IκB complexes also appears to phosphorylate the NF-κB subunits (117).

Phosphatases likely play an important role in the activation of NF-κB, either regulating kinase pathways that may control the signal transduction pathway or by directly dephosphorylating IκB. Based on inhibition by cyclosporin A and on transfection studies, the Ca^{2+}-dependent phosphatase calcineurin is apparently involved in the activation of NF-κB in T cells (75). In addition, FK506, an inhibitor of calcineurin, blocks the activation of c-Rel in B and T cells (118). The target for calcineurin in this pathway is not known. Inhibitors of the ser/thr phosphatases PP1 and PP2A, such as okadaic acid, potently activate NF-κB, suggesting the involvement of a phosphatase in regulating some aspect of the pathway. Evidence that PP2A can directly dephosphorylate IκBα has been provided (T Finco, J Reuther, A Beg, D Ballard, S Shenolikar, A Baldwin, submitted; 119).

5.5.2 CERAMIDE, REACTIVE OXYGEN INTERMEDIATES, AND REDOX STATE Second messengers may be involved in the early activation pathway of NF-κB. The generation of ceramide in response to TNF or IL-1 may be critical in initiating the events leading to NF-κB activation via degradation of IκBα (see 120 and references therein). According to the current model, the interaction of TNF with the 55-kDa TNF receptor activates an acidic sphingomyelinase (Smase) through the generation of diacylglycerol by a phosphatidylcholine-specific phospholipase C. Smase leads to the production of ceramide. That addition of ceramide or sphingomyelinase to cells leads to the activation of NF-κB (see 120) supports this model. Recent evidence indicates that addition of acidic SMase to a cell-free system leads to the degradation of IκBα (121). Based on these data, it has been

proposed that the generation of ceramide leads to the activation of a kinase that phosphorylates IκBα. There is also evidence against a role for ceramide in the activation of NF-κB. Cells deficient in acidic SMase can activate NF-κB in response to TNF or IL-1 (122). Possibly another SMase is involved in the process of activation of ceramide in these cells. Second, inhibition of the ceramide pathway by chronic stimulation of cells with PMA does not inhibit the ability of TNF to activate NF-κB (123). Finally, levels of ceramide that activate the Jun kinase (JNK) are not sufficient to activate NF-κB (124). Clearly more experimentation is needed to resolve whether ceramide is an initiator of NF-κB activation, and, if so, what regulatory pathways exist downstream of this molecule.

Reactive oxygen intermediates (ROIs) have also been proposed to be involved in the activation of NF-κB (4). This is based on the observations that treatment of some cells with H_2O_2 can activate NF-κB and that certain antioxidants such as N-acetyl cysteine or PDTC can block activation of NF-κB by blocking the signal-induced phosphorylation of IκBα. Additionally, many of the known inducers of NF-κB lead to the generation of ROIs. Compelling arguments for the role of ROIs in the activation of NF-κB have been presented by Baeuerle and colleagues (4, 125). These authors propose that the initial response to TNF and other inducers is the production of superoxide anion followed by the generation of H_2O_2 (125). Identification of signaling components that may function downstream of H_2O_2 in the potential activation of NF-κB is lacking. Arguments against ROI involvement in NF-κB activation have been published recently (126, 127) .

Activation of NF-κB binding by thioredoxin, a protein involved in maintaining the redox environment of the cell, involves the reduction of a disulfide bond at cys62 of the p50 subunit (see 128). The sequence of this region is conserved in many of the NF-κB/Rel family proteins. Recently, the structure of thioredoxin and a peptide encompassing the cys62 region has been reported (128). This part of p50 is found in the L1 loop and makes apparently critical contacts with the κB DNA binding site. Expression of thioredoxin increases following exposure of cells to UV light or to H_2O_2 (129), suggesting a possible role in increasing NF-κB activity in response to these inducers.

5.6 *Hypoxia Appears To Activate NF-κB via a Unique Pathway*

Exposure of cells to low oxygen concentrations results in the activation of NF-κB (130), which is likely to be important in situations such as angiogenesis associated with tumorigenesis, and in ischemia. Not surprisingly, activation of NF-κB by hypoxia is correlated with the loss of IκBα. However, an unexpected result was that IκBα is apparently tyrosine phosphorylated in hypoxic cells (130). This would indicate that a kinase is involved in the phosphorylation of IκBα that is distinct from that utilized in responses to TNF, IL-1, LPS, or

PMA. Interestingly, it has been proposed that Ras and Raf, but not Map, kinases are involved in this activation (131). These results need to be followed up, the phosphorylation site(s) mapped, and the functional consequence of the reported tyrosine phosphorylation tested.

5.7 *Activation of NF-κB by Viral Proteins*

Many viral gene products activate NF-κB. This may be advantageous to the virus for several reasons. First, several viruses (such as HIV, CMV, and SV40) have NF-κB binding sites in their promoter/enhancer regions. Second, NF-κB appears to regulate genes required for viral replication. For example, a factor required for the HIV Rev splicing function may be regulated by NF-κB (132). Finally, activation of NF-κB may provide a favorable environment for viral replication, possibly by serving as a mitogenic regulator.

Probably the most intensively studied viral protein known to activate NF-κB is the Tax protein of HTLV-I. Tax has been proposed to activate NF-κB by different mechanisms. One mechanism appears to be through a direct physical interaction with the NF-κB2 100-kDa protein (24, 133, 134). Second, evidence has been provided that Tax activates a signal transduction pathway leading to phosphorylation and degradation of IκB and to the nuclear appearance of NF-κB (90, 135, 136). Finally, Tax may directly interact with NF-κB subunits bound to their target sites and stimulate transcription (137). Activation of NF-κB by Tax is likely to play an important role in the pathogenesis of HTLV-I, possibly through the upregulation of genes encoding IL-2, IL-2Rα, and IL-6 (138). It has been proposed that the HIV Tat protein activates NF-κB (139). Other evidence indicates that Tat potentiates NF-κB activation by TNF by altering the cellular redox state (140). Recent evidence suggests that the κB sites in the HIV LTR are absolutely required for induction of LTR-reporter gene activity in peripheral $CD4^+$ T cells in response to Tat (141). Another important HIV protein, nef, can activate NF-κB when functioning as a cell membrane–associated protein or can inhibit its activation if expressed cytoplasmically (142, 143). A transforming protein of Epstein-Barr virus, the latent membrane protein (LMP-1), has been shown to activates NFκB (144–147). A mechanism for activation may be that LMP-1 associates with factors that are liklely involved with signaling directed by the TNF receptor (148).

5.7.1 CONCLUSION It is unlikely that significant progress will be made on the NF-κB signaling pathway(s) until research proceeds directly downstream from known receptors and directly upstream from the final phosphorylated substrates (IκB and NF-κB subunits). For example, progress in this regard has been made through the identification of a protein associated with the TNF receptor that functions in the signaling of NF-κB activation (149). This protein,

TRADD, interacts with the intracellular domain of the 55-kDa TNF receptor (TNF receptor 1). Overexpression of TRADD leads both to apoptosis and to the activation of NF-κB. However, the pathways for activation of NF-κB and for apoptosis appear to be distinct since crmA, an inhibitor of the IL-1β-converting enzyme, blocked apoptosis but not the activation of NF-κB. At the other end of the pathway, the kinase(s) involved in the phosphorylation of IκB and of NF-κB subunits must be identified. Such data will permit definitive dissection of the signaling pathways involved in NF-κB activation.

5.8 *Inhibitors of NF-κB Activation*

Studies utilizing compounds that inhibit NF-κB activation may provide insight into the activation pathways, may clarify biological situations where inhibition of NF-κB is functionally important, and suggest therapies for diseases related to the acute or chronic activation of NF-κB. An example is tepoxalin, a dual inhibitor of cyclooxygenase and 5-lipoxygenease, which functions to inhibit NF-κB induction by several inducers in multiple cell types (150). Other examples are discussed below.

5.8.1 IL-10 Interleukin-10 (IL-10) inhibits the transcription of a variety of cytokine genes associated with TH1 T cell responses. Recently, IL-10 was shown to inhibit the induction of NF-κB by LPS in human peripheral blood mononuclear cells (151). These data provide an important link in understanding the inhibitory functions of IL-10 toward certain cytokines. It is intriguing to speculate that the mucosal inflammation with abnormal TH1 T cell responses seen in IL-10-null mice (152) is due in part to an inability to block the function of NF-κB.

5.8.2 GLUCOCORTICOIDS, SALICYLATES, AND OTHER IMMUNOSUPPRESSANTS Glucocorticoids are widely used immunosuppressants. For example, prednisone is used to suppress immune responses in organ or bone marrow transplants, and is also used in chronic inflammatory diseases such as arthritis. Glucocorticoids reportedly inhibit NF-κB by two mechanisms. First, several groups report that activated glucocorticoid receptors directly interact with and inhibit activated NF-κB subunits (153–156). A second mechanism involves the transcriptional activation of the IκBα gene in response to treatment with glucocorticoids (157, 158). Glucocorticoids, by upregulating IκBα protein levels, function to block nuclear translocation of NF-κB and DNA-binding. Salicylates (159), which are nonsteroidal anti-inflammatory drugs, inhibit the activation of NF-κB at concentrations used to treat arthritis.

Cyclosporin A (CsA) and rapamycin, both important immunosuppressants that target T cells, inhibit the activation of NF-κB induced in T cells by different

stimuli. CsA blocks the activation of NF-κB in response to the engagement of the T cell receptor (75). One group has reported that rapamycin blocks the induction of NF-κB following CD28 costimulation in T cells (160). Interestingly, this study showed that CD28 signaling caused a sustained inactivation of IκBα. FK506, another inhibitor of calcineurin, blocked the activation of c-Rel in both B and T cells (118).

5.8.3 NITRIC OXIDE Two recent reports indicate that production of nitric oxide (NO) inhibits the activation of NF-κB in endothelial cells (161, 162). The rationale for such a study is that NO inhibits platelet adhesion and smooth muscle proliferation, and modulates leukocyte adhesion to the endothelium. Thus, the production of NO suppresses immune and inflammatory responses. NO inhibits the activation of NF-κB in response to treatment with TNFα (161). The second study showed that NO inhibits the activation of NF-κB and provided evidence that this is through the activation of IκBα (162). Thus NO appears to inhibit activation of NF-κB through a mechanism similar to that described for glucocorticoids (i.e. through the upregulation of IκBα). Since NF-κB transcriptionally regulates the iNOS gene, production of NO may feed back to block its own production through the inhibition of NF-κB. Adding to the complexity, production of NO in lymphocytes reportedly leads to the activation of NF-κB (163).

5.8.4 cAMP As with glucocorticoids and other immunosuppressants, agonists that elevate cAMP inhibit IL-2 expression. This appears to occur through the inhibition of two factors that activate the transcription of IL-2 (164). One of these is NF-κB and the other is uncharacterized and is called the "TGGGC" factor. The inhibition of NF-κB may involve decreased synthesis of NF-κB subunits, and decreased degradation or upregulation of IκB (164, 165). Agonists that increase cellular cAMP levels may be important modulators of immune responses. Prostaglandin E2, an activator of cAMP, may modulate immune function by inhibiting IL-2 secretion on the one hand and activating IL-4 and IL-5 on the other. Thus activators of cAMP may play an important role in the differentiation of TH cells into either the TH1 or TH2 category (164, 165).

6. NF-κB AND DISEASE

Because of its direct role in regulating responses to inflammatory cytokines and endotoxin, the activation of NF-κB may play a role in the development of chronic diseases such as rheumatoid arthritis or in acute situations such as septic shock. Support for a critical role for NF-κB activation in arthritis comes from two observations: NF-κB is activated in the arthritic synovium (S Makarov, A

Baldwin, unpublished), and therapies that are used for treatment, such as prednisone (see above) and gold compounds (166), are now known to block NF-κB.

Septic shock is a systemic inflammatory response that develops when LPS or other microbial products stimulate expression of various inflammatory cytokines. The massive production of these proteins ultimately leads to reduction in blood pressure and to general organ failure. It has recently been proposed that the production of nitric oxide in response to LPS regulates important aspects of septic shock (see 167). These experiments showed that iNOS-deficient mice were protected from septic shock. Since NF-κB activates transcription of the iNOS gene (see above), activation of NF-κB by LPS may play a role in the development of septic shock. Other activators of NF-κB, such as TNFα, may also mediate septic shock (see 168). Autoimmune diseases such as systemic lupus erythromatus (SLE) may involve activation of NF-κB as well (W Jarjour, personal communication). Additionally, Alzheimer's disease may also involve activation of NF-κB in a chronic setting, since the amyloid β peptide causes production of ROIs and activates gene expression through κB sites (169). As described previously, NF-κB plays an important role in the activation of HIV gene expression. The influenza virus protein hemagluttinin activates NF-κB, and this activation may contribute to viral induction of cytokines and to some of the symptoms associated with flu (170). Other examples involving NF-κB and disease pathogenesis are briefly discussed below.

6.1 *Atherosclerosis*

Initiation and progression of atherosclerosis is related to the oxidation of lipids in LDLs. These oxidized lipids become trapped in the extracellular matrix of the subendothelial space and apparently activate NF-κB, leading to transcriptional activation of genes involved in the inflammatory process (171). This process leads to the development of the fatty streak associated with atherosclerosis. Interestingly, mice that are susceptible to atherosclerosis exhibit activation of NF-κB when fed an atherogenic diet (172). Another important contributor to atherosclerosis is thrombin, a serine protease that serves several important roles in actions upon inflammatory cells as well as cells of blood vessel walls. Thrombin likely contributes to the proliferation of the vascular wall that occurs in atherosclerosis and restenosis. Thrombin stimulates the proliferation of vascular smooth muscle cells through the activation of NF-κB (173). Overall, these data provide substantial evidence that NF-κB activation is an important contributor to events involved in atherosclerosis.

6.2 *Oncogenesis*

Evidence for involvement of NF-κB or IκB members in oncogenesis is based on several observations (4, 5): (i) NF-κB proteins are members of a proto-

oncogene family; (ii) the NF-κB2 gene and the *Bcl-3* gene are translocated in certain lymphomas; (iii) NF-κB is activated in quiescent fibroblasts in response to serum growth factors; (iv) NF-κB is activated by viral transforming proteins (Tax and LMP-1, for example; see above); and (v) exposure of cells to IκBα antisense results in oncogenic transformation (174). Additionally, antisense to RelA blocked tumorigenesis induced by Tax in vivo (175). Evidence for a role of translocated NF-κB2 in oncogenesis is based on observations that the normal repressive function of p100 is lost when the ankyrin repeats are deleted in the translocated allele (176, 177). A role for NF-κB/Rel proteins in human cancer presumably would involve transcriptional functions, such as the upregulation of the *c-myc* gene (see above). Conversely, it has been proposed that the activation of NF-κB in macrophages by the antineoplastic agent taxol may contribute to the compound's antitumor properties (178).

6.3 *Ataxia Telangiectasia*

Ataxia telangiectasia (AT) is a human disease characterized by neurological, radiobiological, and immunological deficiencies. Fibroblasts from AT patients are extremely sensitive to ionizing radiation, exhibiting aberrant regulation of DNA synthesis. Recently, a truncated form of IκBα (presumably functioning as a "super-repressor" based on the loss of the critical N-terminal serines) was shown to protect AT cells from killing by ionizing radiation and to correct the defect in DNA synthesis (179). These AT cells express constitutive levels of an NF-κB-like activity. Expression of the truncated form of IκBα reduced NF-κB levels and blocked the radiobiological effects. The gene involved in AT was recently cloned, and the encoded protein is related to yeast lipid kinases TOR1 and TOR2 as well as mammalian PI-3 kinase (180). An additional homology exists with the yeast RAD3 protein, which is involved in cell cycle control. It is proposed that the mutations in the AT gene render it nonfunctional. It is unclear whether NF-κB is activated in other cell types of AT patients and, if so, whether NF-κB may contribute to the neurological or immunological deficiencies. An interesting connection exists between AT patients and the IκBα gene knockout mice (89) in that both exhibit small spleens and thymuses. Thus, inappropriate activation of NF-κB in both cases could cause an immunological defect. The mechanism that relates the loss of function of a PI-3-like kinase/Rad3-like protein to activation of NF-κB is presently unclear.

7. SUMMARY

Ten years of research on NF-κB has led to a much greater understanding of the role of this family of transcription factors and their inhibitors in immunity, inflammation, and cell growth and development. The next challenge is to

untangle the signal transduction pathways involved in controlling activity of NF-κB and to uncover new gene targets for these proteins in immunological and nonimmunological function. Cell type–specific functions for NF-κB, for example in neurons, should be pursued. The functional roles of IκBα and IκBβ, as well as potential new forms of inhibitors, need to be clearly delineated. Additionally, it will be important to understand the exact roles for NF-κB in regulating apoptosis in several situations. Clarification of the mechanisms through which dysregulation of NF-κB contributes to disease is clearly needed. This could promote the development of specific inhibitors of NF-κB and thereby assist in treating certain of these diseases.

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