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Immunologic Tolerance: Collaboration Between Antigen and Lymphokines

G. J. V. NOSSAL

Immunologic tolerance is the process whereby limits are placed on the degree to which lymphocytes respond to an animal's inherent antigens. It is a quantitative rather than an absolute term, as some autoantibody formation is common. Contrary to early hopes, it is not due to some single, simple causative mechanism confined to early developmental stages of the fetal immune system. Rather, self-tolerance results from a variety of complementary mechanisms and feedback loops in the immune system and is thus best seen as part of the general process of immunoregulation.

TWO KINDS OF LYMPHOCYTES, T CELLS AND B CELLS, interact in normal immune responses. Each population consists of cells with different recognition potentials; each cell has only one specificity, conferred on the cell by a surface receptor for antigen. The T cell and B cell repertoires are quite different: the former recognizes small linear peptides in association with certain cell surface molecules, and the latter recognizes conformational determinants on proteins and carbohydrates. The activation of T and B cells by antigen involves not only binding antigen, but also collaborative cellular interactions and regulatory signals delivered by many specific cytokines.

Key results from this and other laboratories support the notion that self-tolerance involves two distinct ways of eliminating from the repertoire high-affinity anti-self T and B cells. For the T cell, an elimination of immature anti-self T cells within the thymus seems to be the chief, but not the sole, mechanism. For the B cell, a downregulatory signaling process that does not involve cell killing appears to occur when immature B cells encounter self antigens. Thus, the repertoire is functionally purged by the process of clonal anergy. The anergic state can now be probed by means of refined single-cell culture techniques and the cloning of cytokines to interact with antigen in lymphocyte stimulation.

Background

Burnet and Medawar were awarded the 1960 Nobel Prize for their discovery of immunologic tolerance (*1*). To understand the capacity of the immune system to distinguish between self and nonself, it is necessary to define three features of immune responses. First, immune recognition depends on specific cell surface receptors that are generated somatically (*2*) by gene translocation mechanisms; each lymphocyte bears on its surface just one combining specificity (*3*). Second, immune responses involve collaboration between two different kinds of lymphocytes: thymus-derived T cells and bone marrow–derived B cells (*4*). T cells mediate chronic inflammation and regulate immune responses by secreting lymphokine factors. The T cell receptor (TCR) for antigen recognizes cell-processed peptide fragments of antigen (*5*) that is noncovalently but firmly associated with an antigen-binding groove on a class I (*6*) or class II (*7*) major histocompatibility complex (MHC) molecule. In other words, the T cell is "blind" to antigen unless it is associated with MHC on a cell surface. B cells are responsible for antibody synthesis. They recognize antigen by immunoglobulin M (IgM) and IgD molecules tethered to the cell membrane. The combining sites of these two isotypes of antibody are identical to each other and to those of antibody secreted by that cell and its progeny. Antibodies recognize conformational determinants of proteins and carbohydrates in free solution. The combining site (about 20 by 30Å) is capable of uniting with 16 amino acids of a protein antigen (*8*). Third, an antigen entering the body must induce lymphocytes specific for that antigen to leave the G_0 state and undergo a series of mitoses. This process, which results in a differentiated clone of immunologic effector cells (*2*), either activated T cells or antibody-forming B cells, is the process of clonal selection. The immunoproliferative cascade is not induced simply by a union of antigen with receptor, but rather requires the cross-linking of multiple receptors, a flux of cytokine growth and differentiation factors, and perhaps other signals dependent on particular forms of cell-cell contact (*9*). In view of the complexity of clonal activation, the responsiveness of a cell to signaling events represents a major variable in immunoregulation (*10*).

The populations of T and B cells of an adult animal represent repertoires of recognition units designed so that most pathogens will elicit both T and B cell responses. Constraints operate on the two recognition systems, however. As regards the T cell system, most typical proteins have only one or two peptides (of a dozen or so amino acids in length) capable of appropriately associating with the MHC molecules of a given animal. The possibility thus exists for nonrecognition of, and therefore genetic nonresponsiveness to, a protein if none of the processed peptides, known as T cell epitopes, fit the particular constellation of MHC gene products of the animal. Clear-cut associations between MHC genotype and susceptibility to

The author is the Director of The Walter and Eliza Hall Institute of Medical Research, P.O. Royal Melbourne Hospital, Victoria 3050, Australia.

infectious diseases are unusual because of the heterozygosity of most individuals in outbred populations at MHC loci and the plurality of loci. Nevertheless, the polymorphism of these loci is usually attributed to selective pressures exerted on a species by pathogens. As regards B cells, the great variation in the affinity constants of antibodies makes it clear that immune recognition is very much a matter of degree, particularly at the initiation of the response. As a result, the "decision" of whether a particular B cell will be triggered by a particular antigen has a large operational component. However, effective immunization is followed by extensive somatic mutation in the genes (called V genes) that code for the component polypeptides of the antibody combining site (*11*). As cells with higher affinity for the antigen are selected for further proliferation, the affinity of the resultant antibody increases progressively.

The vigorous T and B cell responses that follow organ transplantation show that there is no categorical molecular distinction between self and foreign antigens. What is self for individual 1 is clearly foreign for individual 2. Nonresponsiveness to self is somehow learned during the ontogeny of the immune system (*1*), although the frequency of autoantibody production shows that self-recognition is not absolute. Moreover, the capacity to render adult animals permanently nonresponsive to foreign antigens shows that the discrimination between self and not-self can be tricked. So, what cellular and molecular mechanisms underlie immunological tolerance of self?

Four Potential Pathways to Tolerance Induction

In principle there are four ways to achieve self tolerance: (i) ensure that self antigens do not reach the immune system, (ii) delete from the repertoire those cells that react to self antigens, (iii) create a "suppressor" repertoire of cells that inhibit the activation or function of potential anti-self cells, and (iv) provide to immature anti-self cells some pre-signaling that impedes their later response to normally effective stimuli.

These four pathways are not mutually exclusive. Obviously, if an antigen cannot make contact with the immune system, it cannot cause a response. However, sequestration of self antigens behind anatomic barriers constitutes only a small component of self-tolerance. It is applicable to special cases such as proteins of the lens in the eye or to spermatozoal proteins, both of which are strongly immunogenic if injected. But, in view of the extensive traffic of lymphocytes through tissues, and the death, catabolism, and regeneration of many cells, processes that permit self-antigen uptake by infiltrating antigen-processing dendritic cells, a defect in the afferent limb is not the main bulwark against autoimmunity. Failure of antigen presentation may contribute to an absence of autoreactivity. For example, neurons are usually devoid of MHC antigens, and so T cells cannot "see" self antigens on their surface. Upregulation of MHC antigen expression on specialized cells, such as insulin-secreting cells or thyroid epithelium, may be one factor contributing to the pathogenesis of autoimmunity in these organs (*12*). In general, however, tolerance must be a property of the lymphocyte population itself.

Repertoire Purging in Immunologic Tolerance

The articulation of the clonal selection theory (*2*) permitted a precise mechanism of immunologic tolerance to be defined (*13*). This was the suggestion that lymphocytes mature through a stage when any contact with a recognizable antigen leads to death of that cell, a process we have termed clonal abortion (*10*). There is a subtle distinction between this notion and that of clonal deletion. Clonal abortion denotes the elimination of a cell before it has become an immunocompetent member of the peripheral lymphocyte pool. Clonal deletion indicates the elimination of a previously functional lymphocyte. Both must be distinguished from receptor modulation, a process whereby contact with antigen can cause the downregulation of cell surface receptors for antigen. Although this would leave a cell incapable of further response to antigen, it is potentially reversible. For T cells, which develop in the thymus and are then exported into the circulation and the peripheral lymphoid tissues (*14*), clonal abortion would be postulated to occur in the thymus, with a resultant absence of anti-self T cells in the peripheral lymphocyte pool. We showed functional clonal deletion of anti–class I MHC T cells in mice rendered tolerant at birth to foreign MHC antigens (*15*) and obtained evidence that the process was occurring in the thymus. However, our studies could not distinguish between an actual killing of the cells concerned or some other form of functional alteration rendering the cells anergic. There is now evidence that some intrathymic clonal abortion does occur (*16–20*).

The most important TCR is a heterodimer, consisting of an α and a β chain. Each chain has a variable (V) and a constant (C) region. The combining site depends on both V regions, but for certain antigens the β chain appears to be the dominant element. For example, a high proportion of T cells that react with the class II MHC molecule I-E have a TCR containing the V_β segment known as 17a (*16*). In mouse strains that lack the I-E MHC molecule, so have no reason to be tolerant of it, 4% to 14% of splenic or lymph node T cells react with an antibody to V_β17a. In contrast, peripheral T cells from mice that have I-E were only 0.1% positive. Thus, the V_β17a-positive T cells that could react with I-E had in some way been eliminated. However, the results were quite different in the thymus. Here the immature T cells of I-E–positive and I-E–negative mouse strains had equivalent numbers of V_β17a-positive cells. When I-E was present, it caused the elimination of anti–I-E T cells in the thymus during their maturation. This tolerance-inducing, cell-eliminating signal may be delivered by dendritic cells at the cortico-medullary junction in the thymus, past which T cells must traffic before migrating from the thymus (*17*). Presumably, certain self-proteins are processed by these cells into T cell epitopes and presented with class II molecules at the dendritic cell surface. When a reactive lymphocyte passes, it is eliminated by mechanisms that are still obscure. The basic tenets discovered for anti–I-E cells have been confirmed for another self-antigen, Mls^a (*18*).

Transgenic mice have been particularly useful in probing the mechanisms of tolerance induction [reviewed in (*19*)]. The most penetrating example relevant to generation of self-tolerance in the thymus involves an experiment on the male transplantation antigen, H-Y (*20*). This antigen of male mice provokes a T cell attack when male skin or other tissue is transplanted into female mice of the same inbred strain. Kisielow *et al.* (*20*) isolated the α and β TCR genes of a cytotoxic T cell clone that recognized H-Y and injected this DNA into fertilized eggs. The resultant female transgenic mice (which do not have H-Y and therefore are not tolerant of it) expressed the anti–H-Y transgenic receptor in a high proportion of T cells. Most peripheral T cells from such females could be stimulated to proliferate by male H-Y–positive stimulator cells. In contrast, males had no immunocompetent anti–H-Y cells in the peripheral lymphoid tissues. The thymus of transgenic males was drastically depleted of cells. Evidently, the main targets of the clonal abortion process were the many transgene-bearing immature thymocytes in the cortex of the thymus. Another interesting aspect of this problem is that the signal for killing of anti-self H-Y T cells must have involved more

than just a union of the transgenic anti–H-Y receptor and H-Y–bearing cells. An accessory molecule known as CD8, which is expressed on class I–restricted T cells and strengthens the union between a T cell and its target, was also involved. In consequence, certain T cells without CD8 escaped the clonal abortion. A difference between these experiments and earlier ones (*16, 18*) is that the elimination of immature anti-self thymocytes appeared to occur not at the corticomedullary junction but throughout the thymic cortex. The probable reason is that the H-Y antigen is presented in association with class I MHC and class I–positive cells are present throughout the cortex; in contrast, I-E and Mls^a epitopes are class II–restricted and class II–positive cells are sparse in the cortex but abundant at the corticomedullary junction.

While clonal abortion within the thymus may be the main mechanism of self-tolerance in the T cell compartment, at least for some antigens, it is probably not the only one. Anti-self T cells can somehow be silenced in the peripheral tissues as well. This issue is addressed in the section on clonal anergy.

Absolutes are rare in immunology. The lymphocyte repertoire does not "know" what it will be "asked" to recognize, hence the requirement for a highly redundant and degenerate system in which essentially anything can be recognized, more or less well. The corollary is that for an emerging T cell within the thymus there must be some affinity threshold below which clonal abortion does not work. Some relatively low affinity anti-self T cells may escape censorship and could be activated in the periphery. Indeed, when tolerance is induced experimentally, it may not involve a wholesale elimination of the response to the toleragen but rather a subtle repertoire modification, consistent with a residue of a few low-avidity anti-toleragen T cells (*21*). The same question of affinity cutoff points for tolerance induction applies to B cell tolerance and must be addressed regardless of the postulated mechanism.

Suppressor Cells as a Mechanism of Tolerance Induction

A nonimmunologist could be forgiven for being very confused by the literature on suppressor T cells. The initial observation was that some tolerant states could be transferred from tolerant animals to normal animals by the injection of T cells (*22*). This is not in any doubt and occurs in a large variety of experimental circumstances. However, a unique phenotype for suppressor T cells has not been identified, and both T cell clones with specific suppressor activity and well-characterized suppressor factors remain controversial. The best suggestion is that suppressor phenomena are mediated by conventional $CD4^+$ and $CD8^+$ T cells that recognize antigenic determinants (idiotypes) present in the TCRs of anti-self cells (*23*); in other words, suppressor T cells are anti–anti-self. It is worth examining the implications of ascribing most self-tolerance to a repertoire of anti-idiotypic T cells. There are, of course, many self antigens. Their fragmentation would lead to an even larger number of T cell epitopes. These, presumably, associate with self-MHC within the thymus and in other parts of the body. It is already daunting to think of clonal abortion within the thymus having to eliminate so very many potentially self-destructive T cells, although the known high local death rate of thymocytes and the relatively small number of cells exported from that organ are consistent with rigid and extensive clonal censorship within the thymus. If that is not the most important mechanism, one would have to consider the following scenario. Potential anti-self cells leave the thymus, encounter processed MHC-associated self antigens and proliferate, creating an autoaggressive clone. These cells carry peptides derived from their own TCRs and, in turn, stimulate anti-idiotypic T cells, which must then be activated to proliferate before being capable of impeding the anti-self clone. This sequence would have to occur for each and every self antigen. By the same token, when foreign antigens enter the body, some (unstated) mechanism must prevent the development of anti-idiotypic T cells to the T cell clones induced by the foreign antigen. Thus, the concept of the suppressor T cell does not solve the puzzle of self-nonself discrimination, whereas clonal abortion does, since potential anti-self cells are "nipped in the bud" in the thymus. The suppressor T cell is an awkward concept to explain self-tolerance. That is not to say that it may not be correct. However, it is probably an ancillary mechanism to cope with such anti-self T cells as escape the thymus. It may also be an effective mechanism for limiting immune responses to a foreign agent when the foreign agent is not quickly eliminated, as in chronic infections or parasitic infestations and various types of established organ transplants. The demonstration of suppression in various transplantation models ought not obscure the possibility that some repertoire purging has occurred as well, as the two phenomena can coexist (*24*).

B Cell Tolerance and Clonal Anergy

The fourth possible pathway of tolerance induction is to provide a downregulatory signal to potential immature anti-self cells that renders them hyporeactive but does not kill them. There is evidence that this mechanism involves mainly B cells (*25–30*). Since antibody formation by B cells frequently involves the collaborative help of T cells (*4*), the burden of self-recognition is usually placed on the T cell. The existence of tolerance within the B cell compartment has been doubted because of the relative ease of inducing autoantibodies both in vivo and in vitro with artificial B cell stimuli (so-called polyclonal B cell activators), which avoids the need for T cell help [reviewed in (*25*)].

The B cell receptor for antigen consists of immunoglobulin (Ig) molecules as integral membrane proteins; unimmunized mature B cells have both IgM and IgD receptors of identical specificity. Each cell displays a single, unique combining site, which consists of the variable portions of the light and the heavy Ig chains. These V regions result from a series of somatic recombinations of minigenes, namely V, D, and J for the heavy chains, and V and J for the light chains. Each of the minigenes is chosen from an array of germ-line genes. Through this combinatorial gene shuffling, a mouse can generate about 40,000 different heavy chains and about 1,000 different light chains. These numbers are increased still further because of the imprecision with which genes are joined, where nucleotides can be lost or inserted. Through heavy-light chain interaction the potential primary repertoire of B cells therefore consists of at least 10^8 different specificities. Before the process of V gene hypermutation in response to antigenic stimulation occurs (*11*) and greatly enlarges the potential repertoire, an adult mouse, which has about 5×10^8 B cells, may not even have one B cell of each possible kind at any one time. Unimmunized B cells have a life span of only a few days and are replenished at an enormous rate from the bone marrow, so more possibilities will be actualized over a lifetime, but the system has a large stochastic element and a great degree of redundancy.

This raises, with considerable force, the question of what is an antibody. Formal measurement of the affinity of antibody combining sites gives a huge range of $10^{-3}M$ to $10^{-11}M$, although most antibodies would fall in the range $10^{-5}M$ to $10^{-9}M$. When an array of randomly chosen monoclonal antibodies is tested against an array of irrelevant antigens, the number of positive reactions depends on the assay used. If this assay favors low-affinity interactions, the

number of positive reactions will be surprisingly large. For example, when antigen molecules bound to plastic [as in standard enzyme-linked immunosorbent assays (ELISA)] meet IgM antibodies with ten combining sites, antibodies of extraordinarily low affinity can bind. However, the same antigen entering the body might not have stimulated the relevant B cell, because cell surface IgM is only bivalent, not decavalent. Such antibodies may be classified as autoantibodies, even though they are of no pathological significance.

We had an example of this when we probed the B cell repertoire of an unimmunized adult mouse for self-reactive B cells (*26*). The B cells were cultured at limiting dilution in a high-efficiency culture system. They were stimulated with a polyclonal B cell activator, *Escherichia coli* lipopolysaccharide (LPS), and a lymphokine mixture to aid growth and differentiation. After the cells were cultured for 7 days, the supernatants were reacted with methanol-fixed, syngeneic cells in an ELISA assay. About 3% of the activated B cell clones had made antibody that could be classified as an autoantibody by that assay. However, when test circumstances were chosen to force the cells to switch from IgM production to IgG_1 synthesis, and the resultant IgG_1 monoclonal antibodies were examined, only about three of 10^6 B cells scored as self-reactive. The bivalent IgG_1, although of identical specificity to the decavalent IgM, could react with the antigen in only 0.01% of cases. As B cell receptors are bivalent, the autoimmune potential of the remaining 99.99% of cells was a laboratory artifact. The frequency of cells in the preimmune B cell repertoire that express Ig receptors with sufficient affinity to bind self-molecules, and therefore to be potentially triggered by them, is actually very low.

The pathological significance of apparently autoreactive IgM antibodies is further rendered doubtful by the fact that autoantibodies in patients or animals with autoimmune diseases are usually IgG antibodies and, where examined, carry mutations in their V genes. The B cells, therefore, must have been driven by autoantigens (or cross-reactive foreign antigens) to replicate and mature rather than arising through some spontaneous dysregulated activation of unimmunized, unmutated B cells.

B cell tolerance should therefore involve some effect of self antigens on the emerging B cell repertoire in such a way that the number of B cells that respond to accessible autoantigens, and the affinity of the B cells, is less than would be the case to a foreign antigen of similar structure. It does not necessitate a total elimination of all clones with low-affinity anti-self potential. Indeed, too drastic a repertoire purging could result in too great a depletion of the anti-foreign repertoire, in view of the great variety of self antigens. The degree to which self antigens cause B cell tolerance will be influenced by the accessibility and concentration of particular self antigens.

How did we come to the concept of clonal anergy—namely, the existence within the B cell pool of tolerant cells, with receptors specific for the tolerated antigen but incapable of responding to it? Haptenic antigens coupled to a protein carrier at concentrations as low as $10^{-9}M$ could prevent the emergence of immunocompetent anti-hapten B cells from pre-B cells in bone marrow cultures (*27*). We first assumed this was due to clonal abortion, the elimination of hapten-specific cells. We verified that the same process occurred in newborn mice both in vivo and in vitro. When the spleens of young mice that had been rendered tolerant either by intrauterine or neonatal antigen exposure were examined by flow cytometry and other techniques to determine their content of anti-hapten B cells, we found that the number and avidity spectrum of such cells was entirely normal; tolerant cells were present but unresponsive or functionally silenced (*28*). When pre-B cells matured in vitro in the presence of antibody to the μ chain of Ig at very low concentration, B cells developed that had a normal density of surface Ig but that failed to respond to mitogenic signals. A whole population of anergic B cells can therefore be generated (*29*). This finding raises a number of questions. What is the lesion in the tolerant cell? Can it be reversed? Is the anergy complete or partial? Is it equally profound for all signals? Can anergy also be induced in adult cells under some circumstances, or is it confined to immature B cells?

To address these questions, it is first necessary to analyze the various steps involved in B cell activation, division, and differentiation. Before doing so, however, a recent experiment which strongly supports clonal anergy should be described.

Functional Silencing of Self-Reactive B Cells in Transgenic Mice

Goodnow *et al.* (*30*) created two kinds of transgenic mice. The first carried the hen egg lysozyme (HEL) gene exons linked to the metallothionein promoter. Such mice produced up to 28 ng of HEL per milliliter from a very early stage of life, even without zinc exposure, and both T and B cells were tolerant to HEL. The second set of mice were transgenic for the rearranged VDJ genes of the heavy chain gene of a high-affinity antibody to HEL; the transgene constant region gene segment contained both μ and δ constant regions. Rearranged light chain genes from the same hybridoma were also introduced. Mice that integrated both heavy and light chain genes were selected. About 90% of the splenic B cells of such mice expressed the transgenic anti-HEL Ig receptor, both in the IgM and the IgD form. Such mice were found to have spontaneous, high serum titers of antibody to HEL and an excessive anti-HEL response.

Next, the two kinds of mice were mated to create doubly transgenic mice bearing both antigen and antibody transgenes. In these mice, the large number of transgene-expressing B cells were not deleted. However, the B cells were profoundly anergic, failing to respond to either T cell–dependent or T cell–independent mitogenic stimuli. One interesting feature of these anergic B cells is that they had an abnormally low ratio of IgM to IgD. This was in contrast to the B cells of mice expressing only the antibody transgenes, which had the high IgM-IgD ratio characteristic of B cells newly exported from the bone marrow. The selective loss of surface IgM in the doubly transgenic mice was an unexpected finding and is so far unexplained. The total number of transgene-expressing B cells in the spleens gradually decreased with advancing age, consistent with our finding of a shortened life span for anergic B cells (*29*). This model offers many opportunities for a detailed molecular and cellular analysis of clonal anergy.

Another model of B cell tolerance in transgenic mice was reported by Nemazee and Bürki (*31*). Transgenes for the μ heavy and κ light chains of an antibody to $H\text{-}2^k$ class I MHC were introduced. In $H\text{-}2^d$ transgenic mice, 25 to 50% of the B cells expressed the transgenic IgM receptors, and serum antibody to $H\text{-}2^k$ is readily detected. In $H\text{-}2^d \times H\text{-}2^k$ hybrid transgenic mice, B cells expressing the transgene could not be detected in either the bone marrow or the spleen. The results were consistent with clonal abortion of transgene-expressing B cells as they encountered the plentiful $H\text{-}2^k$ antigens on surrounding cells in the marrow. However, a modulation of surface receptors and a resultant anergic state could not be completely ruled out. It is likely that early exposure to multivalent antigen could have delivered a signal akin to a very high concentration of antibodies to the μ chain, with resultant prevention of emergence of Ig receptors. Only study of the possible reversibility of the lesion will reveal definitively whether abortion or anergy is at work.

Collaboration Between Antigen and Lymphokines in B Cell Signaling

The activation of B cells from the G_0 state to blastogenesis, sequential mitoses, differentiation to IgM formation, and eventually to a clone capable of forming multiple antibody isotypes other than IgM is a complex and highly regulated process. The chief factors influencing this process are (i) engagement of the B cell surface Ig by antigen in such a way that the receptors are cross-linked, either because the antigen is itself multivalent or because multiple molecules of the antigen are associated with the surface of an antigen-presenting cell; (ii) conjugation of the B cell with a T cell that has been activated, usually to some epitope of the antigen distinct from the epitope recognized by the B cell; (iii) a directed flow of lymphokines from the T cell to the B cell across a synapse-like junction; and (iv) modulatory influences of other cytokines in the extracellular milieu, derived chiefly from macrophages and T cells. These events occur in vivo in distinct microenvironments within lymphoid tissues, where various kinds of antigen presenting cells serve to bring trafficking lymphocytes into proximity. Detailed analysis of the processes in vitro can, at best, reveal only part of the story.

The large body of work on B cell activation from many centers has been reviewed (*9, 32*). My laboratory uses a particular culture system to provide a precise and orderly analysis of the overlapping stages in B cell activation (*33*). The B cells are pre-fractionated by reactivity with a particular hapten, usually fluorescein (fl). Single fl-specific B cells are cultured in 10-μl Terasaki wells, which permits periodic microscopic examination. Cloning efficiency can be increased by addition of 3T3 fibroblasts. The B cell is stimulated by fluorescein conjugated multivalently to a carrier molecule, such as polymerized flagellin (fl-pol). In addition, one or more cloned cytokines are added to the culture medium. The immunoproliferative process is categorized into four stages. First, activation of the B cell out of the G_0 state is measured by microscopic observation of enlargement of the cell (*34*). Second, division is observed microscopically by recording increasing cell numbers. Third, IgM secretion is assayed by subjecting culture supernatants to an ELISA. Fourth, switching to downstream isotypes, particularly IgG_1, is monitored by holding the cultures somewhat longer and performing the ELISA with isotype-specific antibody-enzyme conjugates.

The chief results for normal fl-specific B cells were as follows. The most efficient way to drive the cell out of G_0 and cause it to enlarge was to combine fl-pol with interleukin-4 (IL-4), although either IL-4 or fl-pol alone was also partially effective. Cells stimulated with fl-pol plus IL-4 could divide to a limited extent but were not able to differentiate to antibody-forming clones. The most effective growth and differentiation factor, acting as a single agent, was IL-5, as cells stimulated with fl-pol plus IL-5 divided and formed significant amounts of IgM. IL-5 alone was totally ineffective; it required the early signals conferred on the cell by fl-pol. Cells stimulated with fl-pol plus IL-5 could not progress to the formation of significant quantities of isotypes other than IgM. As had previously been reported by others (*9, 32*), IL-4 proved to have a second, powerful action. While IL-4 alone could not induce antibody formation, in the presence of fl-pol plus IL-5, it caused massive isotype switching, predominantly to IgG_1 synthesis. IL-6 also had some, albeit modest, effects in this system, promoting somewhat greater Ig synthesis without affecting cell division.

The role of IL-2 in B cell activation is fascinating, if somewhat complex. Acting alone on small, resting B cells, IL-2 is ineffective, but combined with fl-pol it is moderately active in driving B cells to division and IgM antibody synthesis (*35*). It is known that G_0 B cells lack IL-2 receptors, so it appears that fl-pol acting on fl-specific cells can moderately upregulate IL-2 receptors. We therefore embarked on a separate study of the induction of IL-2 receptors on B cells (*36*). The IL-2 receptor is a heterodimeric molecule that consists of a 75-kD α chain with intermediate affinity for IL-2, a β chain with low affinity for IL-2, and the heterodimer itself expressing high affinity for IL-2. The signal transduction appears to be mediated via the α chain. B cells stimulated by LPS alone do not display IL-2 receptors. B cells stimulated with IL-5 are induced to express the β chain of the IL-2 receptor only. They are unresponsive even to high concentrations of IL-2, as the β chain has no signal-transducing capacity. B cells stimulated with IL-4 are induced to express the α chain of the IL-2 receptor only. They respond to IL-2, but only at relatively high concentrations. B cells stimulated by IL-4 plus IL-5 express the heterodimeric high-affinity receptor and hence are stimulated by low concentrations of IL-2. This appears to be the first example of two chains of a single receptor being separately regulated by two different cytokines. Though work on the synergy between IL-4, IL-5, and IL-2 in B cell signaling is still in progress, it is already clear that if the former two are used in relatively low concentrations, IL-2 itself at low concentration can have a marked synergistic effect both on growth of clones and on antibody formation. It is therefore possible that, in a physiologic setting, multiple cytokines guide B cell clonal development.

Clonal Anergy and B Cell Signaling

Fluorescein-specific B cells can also be harvested from animals made tolerant to fluorescein either in utero or neonatally. Provided that limitingly small amounts of toleragen are used to induce

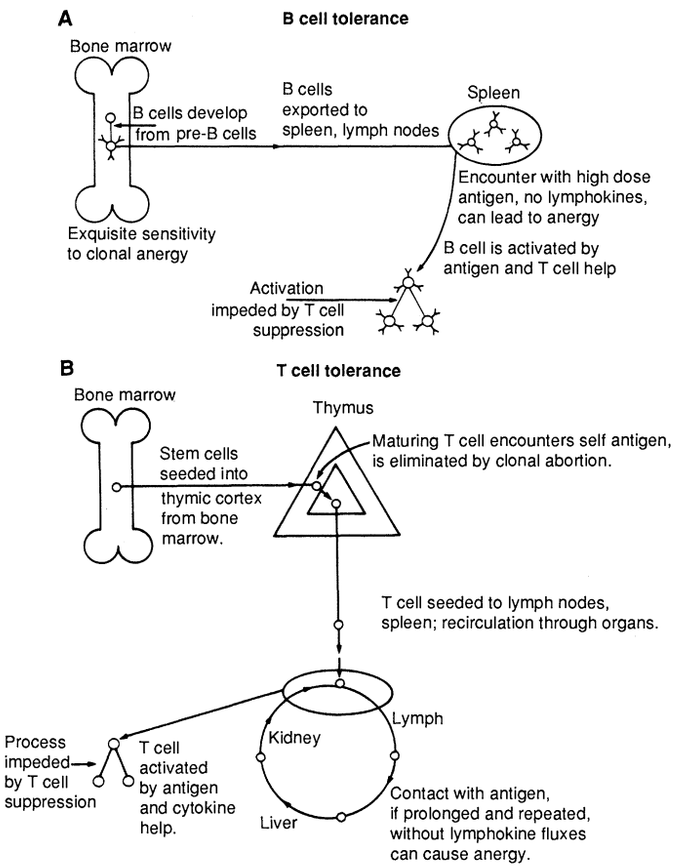

Fig. 1. Potential steps in lymphocyte differentiation for tolerance induction in (**A**) B cells and (**B**) T cells.

tolerance, clonal anergy in these single cells is not complete, as analyzed by this precise, four-stage assay. In particular, signals that do not involve crosslinking of the Ig may be followed by normal early steps (*33, 37*). Thus, IL-4 or LPS cause activation and some cell multiplication in the anergic cell. However, correct differentiation to antibody-forming status is impeded. For example, when a strong stimulus like fluoresceinated LPS plus an IL-4–containing lymphokine mixture is used, IgM secretion is substantially reduced and the switch to IgG_1 is almost completely prevented. It appears that the further the cell is prodded down the immunoproliferative cascade, the fewer cells make it. It is of great interest to determine how the transgenic B cells in HEL–anti-HEL doubly transgenic mice behave when analyzed in the single cell assay. Furthermore, anergic B cells can be produced by treatment with antibody to μ chain, which yields a larger cell sample than can be obtained from (the rare) antigen-binding cells. Such models should be useful for determining the biochemical basis of the anergic state.

Clonal Anergy and Peripheral T Cells?

In experiments in which transgenic mice were constructed with class II (*38, 39*) or class I (*40*) MHC genes, the rat insulin promoter guided expression of the transgene exclusively in the insulin-secreting β cells of the pancreatic islets of Langerhans. Such animals became diabetic at a very young age, whether the transgene was syngeneic or allogeneic to the mouse in question. Apparently overexpression of MHC molecules leads directly to the death of β cells by a nonimmunologic mechanism. When an allogeneic transgene was expressed, there was no lymphocytic infiltration of the islets. The mice appeared to be tolerant of the transgene product, even though it was expressed only in the pancreas and not at all in the thymus. In the class I model, there was no islet cell infiltration even after immunization of transgenic mice with allogeneic spleen cells of the same MHC genotype as the transgene. Mixed lymphocyte cultures and cytotoxicity tests also substantiated that the T cells in the periphery of such mice were tolerant of the transgenic MHC. However, thymus cells themselves from the transgenic mice were not tolerant. The tolerance had, in this case, not been due to intrathymic clonal abortion, but had apparently been acquired peripherally. Furthermore, the tolerance waned with time as the diabetes became more severe, perhaps indicating that maintenance of peripheral tolerance required continued presence of the antigen. When all the β cells have been destroyed, no more transgene product is manufactured, and as further competent cells emerge from the thymus, normal alloreactivity gradually reasserts itself in the periphery. A clonal anergy model best explains these observations.

In a different type of transgenic model, a class I MHC transgene was placed under the control of the zinc-inducible metallothionein promoter (*41*). The transgene was expressed in many tissues, including liver, kidney, and the exocrine portion of the pancreas. Zinc sulfate injections increased transgene expression. The thymus showed only low amounts of transgenic MHC. In contrast to the β cells above, the cells of these mice showed no harm from MHC overexpression. There was no lymphocytic infiltration of any organ—that is, the mice were operationally tolerant of the transgene. Yet, in vitro tests showed T cell responsiveness to the apparently tolerated MHC transgene product. When transgenic mice were treated with x-rays to destroy the bone marrow and lymphoid cells, and then infused with mature, syngeneic T cells from normal (nontransgenic) mice, inflammatory lesions and cell necrosis resulted, but the intensity of this graft-versus-host attack decreased with time. Perhaps a certain degree of peripheral tolerance (clonal anergy?) developed with time. These new results are consistent with an earlier report (*42*) in which lymphocytes were injected intravenously into class I–incompatible recipients, and 1 to 2 weeks later the host cells had somehow rendered the donor T cells tolerant to the host class I MHC antigen and the donor cells had rendered the host T cells tolerant to donor class I antigen. The data were interpreted to imply that self-reactive T cells were "vetoed" by other lymphocytes, including cytotoxic T cells, acting as targets. This peripheral tolerance induction may be a fail-safe mechanism to back up intrathymic clonal abortion.

Whatever the mechanism of acquisition of this type of peripheral T cell tolerance, it is clear that when mature B cells are exposed to a sufficient concentration of a multivalent antigen for a sufficient time, they too can be rendered anergic. However, this requires far higher concentrations than are effective for immature cells (*10*). It seems fair to conclude that both T and B cells can develop a state of anergy if exposed to antigen in the absence of costimulatory signals; this anergy does not necessarily involve receptor modulation or cell death.

The possible steps in lymphocyte differentiation at which tolerance can be induced are schematically represented in Fig. 1.

Conclusions

Lymphocyte responses to antigen are exquisitely regulated. The basic thrust of our research has shown that downregulation—that is, the induction of a refractory state—is as important as activation in lymphocyte physiology and forms an important component of self-tolerance. Indeed, even in the analysis of lymphocyte activation, there is evidence that lymphocytes can "remember" past signals. For example, a B cell stimulated with IL-4 "knows" to switch to IgG_1 production, given appropriate further signals, even if the IL-4 is removed from the medium well before isotype switching. In addition, we observed that immature lymphocytes are particularly sensitive to negative signaling, a teleologically sensible fact, since self antigens are always present and can thus "catch" the cell before it has become competent, whereas foreign antigen is pulsed in unexpectedly, thus encountering many cells that have passed the most tolerance-sensitive phase. Finally, there is a major quantitative element to clonal anergy: the higher the toleragen concentration or the affinity of the cell for the antigen concerned (or both), the stronger is the negative signal. If anergy does shorten the life span of the affected cell, then the concepts of clonal anergy and clonal abortion essentially merge into one another.

There is now evidence that repertoire purging within the thymus—that is, clonal abortion—is the main tolerance mechanism for T cells with respect to at least some antigens. Transgenic studies further support functional repertoire purging (clonal anergy) as a major mechanism for B cells and probably for T cell tolerance. The most plausible mechanism of action of T cell suppression is to regard the target for suppression as idiotypic epitopes on anti-self cells. Anti-idiotypic suppressor T cells may play their chief roles in limiting anti-foreign immune responses and as a fail-safe mechanism against anti–self-reactivity induced in cells that have, for whatever reason, escaped the repertoire-purging mechanism of clonal abortion and clonal anergy.

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