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The Need for Central and Peripheral Tolerance in the B Cell Repertoire

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The immune system normally avoids producing antibodies that react with autologous ("self") antigens by censoring self-reactive T and B cells. Unlike the T cell repertoire, antibody diversity is generated within the B cell repertoire in two phases; the first occurs by gene rearrangement in primary lymphoid organs, and the second phase involves antigen-driven hypermutation in peripheral lymphoid organs. The possibility that distinct cellular mechanisms may impose self tolerance at these two different phases of B cell diversification may explain recent findings in transgenic mouse models, in which self-reactive B cells appear to be silenced both by functional inactivation and by physical elimination.

ANTIBODIES HAVE BECOME POWERFUL TOOLS IN RESEARCH and biotechnology. In these areas, as in the acquisition of immunity, production of potentially useful antibodies often depends on two aspects of self-nonself discrimination during the antibody response. First, antibodies are not normally produced against self antigens, and secondly, antibodies to foreign antigens are normally directed exclusively at regions on the foreign antigen that differ from self. The physiological imperative of avoiding autoantibody production has been appreciated since the first demonstration in 1900 of the destructive effects of isoantibodies on genetically mismatched red blood cells (*1*) and the resultant possibility that "formation of autotoxins [autoantibodies] would . . . constitute a danger threatening the organism more frequently and much more severely than all exogenous injuries" (*2*, p. 253). The implications of self tolerance in research and biotechnology are equally profound, since the absence of antibodies directed to self antigens makes it relatively straightforward to generate species-specific antibodies for use in sensitive immunoassays or allele-specific antibodies for blood grouping and tissue typing before transfusion or organ transplantation. Nevertheless, the immunological mechanism responsible for preventing autoantibody production remains controversial.

Historically, self tolerance has long been thought to involve a mirror image of the processes involved in immunity (*3, 4*). Rather than being genetically determined characteristics, both immunity and tolerance were found to be acquired during development of the immune system (*3, 5*). Not surprisingly, the concept that immunity was acquired by "clonal selection" of foreign antigen–specific precursor cells and their differentiation into antibody-secreting cells (*4, 6, 7*) led to the hypothesis that tolerance, as a mirror image, would be acquired by "clonal deletion" or functional inactivation ("clonal anergy") of precursor B lymphocytes that expressed antibodies to self antigens (*4, 8, 9*).

The discovery of T and B lymphocytes (*10*) has necessitated a reappraisal of the validity of this simple model for preventing production of autoantibodies. Since antigen-specific B cells need to collaborate with antigen-specific T cells to mount efficient antibody responses to foreign antigens (*10, 11*), the failure to produce antibodies to self antigens could merely reflect clonal deletion of self antigen–specific T cells rather than any change in the B cells themselves (*12*). However, T cells and B cells collaborate in such a way (*13*) that foreign antigen–specific T cells may, in fact, interact with self-reactive B cells whenever a foreign antigen becomes noncovalently associated with a self antigen, as in the interaction between a viral DNA-binding protein and self DNA (Fig. 1A) or when a foreign antigen happens to cross-react with a self antigen, for example, during the production of species-specific or allele-specific antisera (Fig. 1B). The absence of high-affinity autoantibodies to self antigens in these situations (*14*) therefore indicates that during acquisition of humoral immunity self tolerance not only involves T cell unresponsiveness but also processes that act directly on the B cells. In this article, we focus on the issue of tolerance within the B cell repertoire, with particular emphasis on the similarities and differences to T cell tolerance and on recent work in transgenic mice.

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Self Tolerance in B Cells Is Not Absolute

When T cell tolerance is bypassed by immunization with a self antigen coupled to a foreign carrier (Fig. 1A) production of autoantibodies is not entirely prevented by self tolerance in the B cell repertoire. An example of partial "breakage" of B cell tolerance in such a situation is shown in Fig. 2A, in mice that express hen egg lysozyme (HEL) as a transgene-encoded self antigen. Lysozyme-transgenic mice immunized with lysozyme on its own do not produce antibodies to lysozyme because both T and B cells are tolerant (*15*). Identical transgenic mice immunized with lysozyme coupled to a foreign carrier, sheep red blood cells (SRBC), however, mount a small but significant antibody response to lysozyme as measured by serum antibody titers, albeit at a much lower level than in nontransgenic littermates (*15*). Moreover, while the number of plasma cells secreting antibody to lysozyme is also generally reduced in the transgenic mice, their frequency does on occasion approach that found in nontransgenic controls (Fig. 2A), suggesting at face value that there may be little or no tolerance to lysozyme within the B cell repertoire. Nevertheless, when the affinity of the autoantibodies secreted by the nontolerant clones of anti-lysozyme B cells is estimated (Fig. 2B), it is approximately 1/100 that of the antibodies to lysozyme produced by the nontransgenic mice, indicating that only the higher affinity B cells become tolerant to lysozyme. In other words, the failure to induce tolerance in low-affinity self-reactive B cells does not imply a complete lack of self tolerance in the B cell repertoire, but rather, provides a clue to its mechanism by suggesting that self-reactive B cells may need to bind a critical threshold of self antigen in order to be rendered tolerant.

The existence of a binding threshold for tolerance induction, determined by self antigen concentration as well as by B cell affinity, is further supported by the failure of even high-affinity anti-lysozyme B cells to be rendered tolerant in transgenic mice expressing very low concentrations of lysozyme (*16*). Similarly, classical models of "induced" tolerance to exogenous antigens have also demonstrated a requirement for a critical dose of antigen and selective silencing of higher affinity B cells (*17, 18*). In physiological terms, the failure to induce tolerance in low-affinity anti-self B cells to many self antigens (*14, 19*) appears to pose little risk of autoimmune disease in most cases, presumably because the titers and affinity of such antibodies are normally below those needed to initiate tissue destruction or to interfere with biological functions. For similar reasons, low affinity anti-self antibodies generated during the production of species-specific or allele-specific antibodies do not normally complicate immunoassays or tissue typing.

In a sense, the incomplete nature of B cell self tolerance parallels recent findings in T cell tolerance, which indicate that tolerance is achieved in strongly self-reactive T cells by clonal deletion within the thymus (*20, 21*), whereas T cells thought to bind only weakly to self major histocompatibility complex (MHC) antigens escape tolerance and in fact are positively selected to become self MHC–"restricted" T cells (*21, 22*). Mechanistically, however, silencing of high-affinity B cells must involve somewhat different processes, since development of a high-affinity B cell repertoire depends on fundamentally different molecular and cellular events.

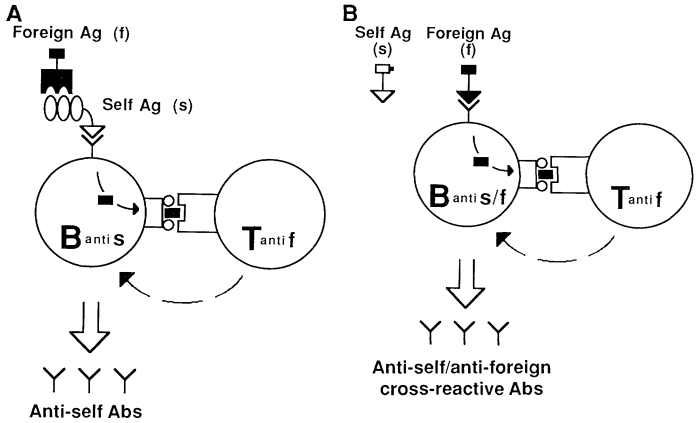

Fig. 1. Two situations where collaboration between self-reactive B cells and foreign antigen–specific T cells can occur, potentially resulting in production of autoantibodies [adapted from (*14*) with permission, © 1990 Cold Spring Harbor Press]. B cells specific for foreign antigens normally collaborate with anti-foreign T cells by internalizing foreign antigens bound to cell surface Ig and then presenting peptide fragments of the antigen complexed with self-MHC molecules for recognition by the TCR on anti-foreign helper T cells. The T cell, in turn, is triggered to release lymphokines or other mediators that stimulate proliferation and differentiation of the B cell (*11, 13*). In the absence of any mechanism for direct silencing or elimination of self-reactive B cells, however, these cells could also collaborate with anti-foreign helper T cells as a result of either (**A**) formation of complexes between a foreign antigen and a self antigen, such as viral DNA-binding proteins bound to self DNA; or (**B**) presence of regions on a foreign antigen that happen to bear structural homology to a self antigen and thus cross-react with self, as occurs when animals are immunized with allogeneic or xenogeneic proteins or cells. In both situations, internalization of the complex and presentation of foreign peptide fragments by the self-reactive B cell will trigger anti-foreign T cells, thus giving rise to a potentially pathogenic autoantibody response and making it very difficult to produce allele-specific or species-specific antisera or monoclonal antibodies. A more detailed discussion can be found in (*14*).

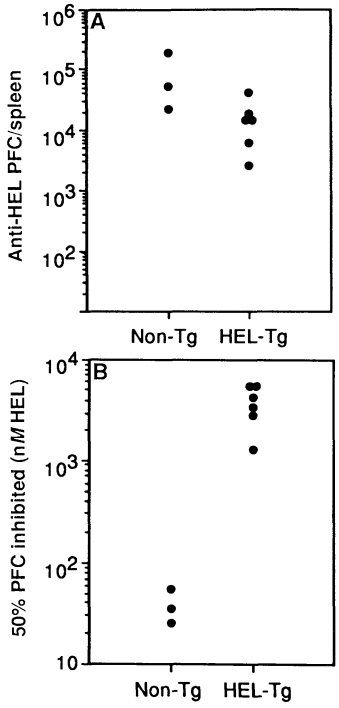

Fig. 2. Incomplete self tolerance in the B cell repertoire due to the failure to induce tolerance in low-affinity B cells. Transgenic mice carrying a gene construct encoding hen egg lysozyme (HEL) under transcriptional control of the mouse albumin promoter (*14*) express lysozyme as a new self antigen, resulting in tolerance to lysozyme within both the T and B cell compartments (*15, 41*). The functional absence of lysozyme-specific helper T cells, due to tolerance, can be circumvented by challenging the mice with a conjugate of lysozyme coupled to foreign (sheep) red blood cells (SRBC), potentially creating a situation where lysozyme-specific B cells can collaborate with SRBC-specific helper T cells. (**A**) In this particular experiment, the number of cells induced to secrete IgG anti-lysozyme antibody [plaque-forming cells (PFC)] in individual lysozyme transgenic mice, as indicated by dots, is similar to that in nontransgenic littermates, demonstrating that a significant number of lysozyme-specific B cells are not tolerant. (**B**) However, when the relative median affinity of the antibodies is estimated by measuring the concentration of free lysozyme required to inhibit formation of 50% of the plaques (*15*), 100 times higher concentrations of lysozyme are needed to inhibit PFC from the transgenic mice compared with nontransgenic controls. Thus, B cells with higher affinity for lysozyme are either absent or incapable of responding in the lysozyme transgenic mice, while low-affinity cells are still capable of mounting a residual response.

Differences in Development of T and B Cell Repertoires

Both T cells and B cells recognize foreign or self antigens through evolutionarily related cell surface receptors, the T cell receptor (TCR) and antibody (immunoglobulin, Ig), respectively (*23, 24*).

Both forms of receptor are comprised of two discrete polypeptides, the α and β chains in the T cell receptor and the heavy (H) and light (L) chains of immunoglobulin, although the stoichiometry differs. The antigen-binding site of Ig molecules, and probably of the TCR, is formed primarily by the juxtaposition of six hypervariable polypeptide loops (complementarity-determining regions, or CDRs), three of which are encoded within each NH_2-terminal variable (V) domain of paired heavy and light chains or paired α and β chains. The individual CDRs are numbered (CDR1, CDR2, and CDR3) as they appear within the primary sequence of each variable domain, with CDR1 being closest to the NH_2-terminus. While the overall layout of the two kinds of receptor appears similar, there are important differences in the ligands recognized by Ig and TCR molecules and in the mechanism whereby the V domains are diversified. The way in which the receptor repertoires are formed, in turn, affects the way self tolerance can be imposed.

The TCR on T cells recognizes a complex of self-MHC molecules and peptides derived from either self or foreign antigens (*24, 25*). As hypothesized by Davis and Bjorkman (*24*), it seems likely that CDR1 and CDR2 of the TCR, which display only moderate diversity between different T cells, contact the MHC component of the ligand, which is itself only moderately variable. By contrast, CDR3 of the TCR exhibits extraordinary sequence diversity, as does the range of peptides with which it is thought to make contact. Not only does this pattern of TCR sequence diversity make sense in terms of the diversity of the ligand it recognizes, but it can be generated entirely by somatic rearrangement of variable (V), diversity (D), and joining (J) TCR-gene segments (VDJ recombination) within the thymus, since CDR1 and CDR2 are encoded within a relatively small number of germline V_α and V_β gene segments, whereas CDR3 is encoded at the VJ_α or VDJ_β junctions where the sequence diversity approaches randomness (*24*). Because the entire repertoire of TCR specificities is generated within the thymus and the TCR sequence on a given T cell does not change once that cell has emigrated from the thymus (*24, 26*), T cells reacting strongly with self antigens will arise mainly in this site (Fig. 3). Not surprisingly, self tolerance in the T cell repertoire is therefore imprinted predominantly by "central" mechanisms of clonal deletion or inactivation acting within the thymus (Fig. 3) (*20, 21*). "Peripheral" mechanisms of T cell tolerance may only be needed for those organ-specific self antigens that are not encountered during T cell development within the thymus (*27*).

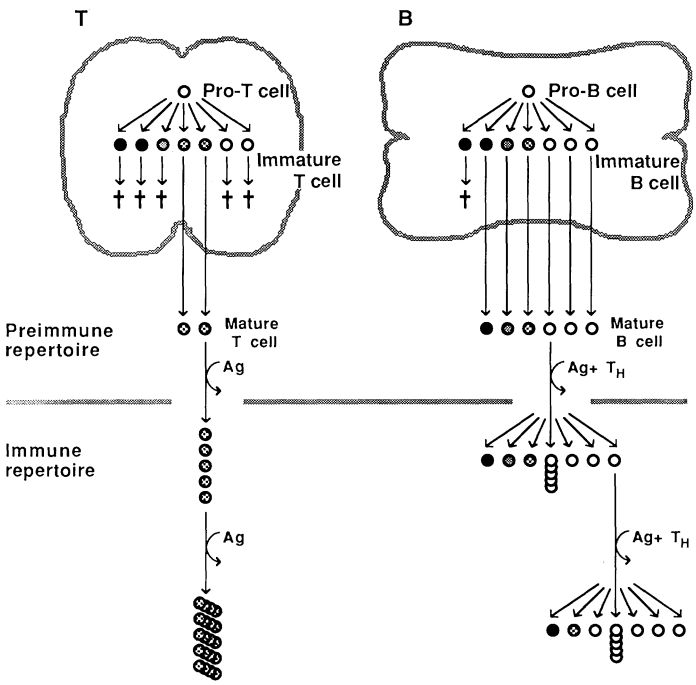

Fig. 3. Receptor diversification in mammalian T and B cell repertoires. The radiating arrows indicate diversification of antigen receptors, filled circles denote strongly self-reactive T or B cells, and shaded circles denote weakly self-reactive cells. In T cells, essentially all receptor diversification occurs by VDJ recombination within the thymus, and consequently, all self-reactive T cells will arise and most will be deleted in this "central" lymphoid organ. By contrast, receptor diversification in B cells occurs in two phases, first by VDJ recombination within the bone marrow to generate a predominantly low-affinity preimmune repertoire, and subsequently by hypermutation in mature peripheral B cells, thus giving rise to an immune repertoire of predominantly high-affinity antibody-producing clones. Strongly self-reactive B cells can develop during both phases of receptor diversification, either from Ig-negative pre– or pro–B cells during VDJ recombination or as a result of hypermutation in previously nontolerant B cells with only weak or no self reactivity. In order to avoid production of pathogenic autoantibodies, self tolerance must therefore be imposed not only in immature B cells within central lymphoid organs but also in mature B cells in the periphery.

Development of the B cell repertoire is quite different. By contrast with the TCR, antibodies appear capable of binding to any ligand with a defined structure, including proteins, carbohydrates, nucleic acids, and small molecules or side chains. Moreover, antibody-antigen interactions must frequently be of high affinity to be effective, since antibody monomers need to bind relatively irreversibly in order to neutralize viruses and toxins or to opsonize larger microorganisms (*28*). The structural basis for high-affinity antibody binding, at least to protein antigens, appears to involve a "lock and key" mechanism whereby near perfect shape complementarity between the surface of the protein and the antigen-binding site of the antibody provides an extensive interface for various forms of bonding (*29, 30*). Since the antibody residues that form contacts with this diverse range of antigens are derived more or less equally from all three CDRs of the heavy and light chains (*29, 30*), it is therefore necessary for the repertoire of antibody molecules to exhibit much greater diversity in CDR1 and CDR2 than that found in the TCR repertoire.

To meet the need for more extensive diversity in CDR1 and CDR2, both mammals and birds (*31*) have evolved additional mechanisms for generating antibody diversity not found in the TCR repertoire. Thus, in mammals, the antibody repertoire is generated in two phases (Fig. 3). First, a "preimmune" repertoire of B cell clones expressing predominantly low-affinity antibodies (*32*) is generated by VDJ recombination in the bone marrow and fetal liver (*33*). Secondly, after contact with foreign antigens and helper T cells in peripheral lymphoid organs, individual B cell clones are selectively expanded, and their receptors are further diversified by a poorly understood process of somatic hypermutation (*32, 34*), which introduces point mutations throughout the V_H and V_L regions and thus generates variability in all three CDRs. Iterative cycles of hypermutation and selection appear to account for "affinity maturation" of the antibody response (*32*), thereby giving rise to an immune repertoire of high-affinity antibodies (Fig. 3).

The fact that the mammalian B cell repertoire, in contrast to the T cell repertoire, is only partly formed by VDJ recombination within primary lymphoid organs has important implications for self tolerance. Since most immature B cells arising by VDJ recombination in the preimmune repertoire express Igs with low affinity for most antigens (*32*), self-reactive cells at this stage of B cell development are unlikely to bind sufficient levels of self antigen to be rendered tolerant, with the exception of those self antigens that are present at very high concentration or in multivalent form. Conversely, failure to induce tolerance in low-affinity self-reactive B cells in the preimmune repertoire may pose little risk of autoimmune disease unless they react with multivalent self antigens such as cell surface

molecules, in which case cooperative binding of bivalent or multivalent secreted antibodies can compensate for low intrinsic antibody affinity. In addition to low affinity, the specificity of antibodies expressed by immature B cells in the preimmune repertoire may bear little resemblance to specificities expressed by hypermutated progeny in the immune repertoire (Fig. 3). For example, hypermutation can either result in a nonself-specific B cell acquiring a new autoreactive specificity (*35*) or convert a nontolerant B cell with low affinity for self antigen into a high-affinity potentially pathogenic autoantibody-producing B cell clone (*36*). Given the propensity for collaboration between autoreactive B cells and foreign-specific T cells (Fig. 1), it becomes essential for B cell tolerance to be imprinted not only by central mechanisms within the bone marrow, but particularly by peripheral mechanisms with the potential to silence mature B cells following hypermutation in secondary lymphoid tissues.

Models of Self Tolerance in Ig Transgenic Mice

The extraordinary diversity of specificities within the B cell repertoire makes direct study of the mechanisms of B cell tolerance difficult for two reasons. B cells specific for particular antigens, including self antigens, arise at very low frequencies during B cell development and cannot be readily detected by conventional techniques. Second, low-affinity antigen-specific B cells tend to arise more frequently than cells with high affinity, since the probability of producing a receptor with an approximate fit to a particular antigen is higher than that of producing a perfectly fitting receptor. Thus the presence of such cells complicates studies aimed at following the fate of higher affinity, potentially pathogenic B cell clones.

Transgenic mice carrying Ig genes that are already functionally rearranged to encode a particular antibody specificity provide a unique opportunity for studying the development and selection of antigen-specific B cells in vivo, since many of the B cells arising in such mice express a homogenous antibody specificity. This uniform specificity arises because most of the B cells express the transgene-encoded antibody and because the rearranged transgenes activate a feedback mechanism of "allelic exclusion" to prevent rearrangement and expression of the animal's own endogenous Ig genes (*37*). As a consequence, the only Ig molecules expressed by most of the B cells are transgene-encoded (*15, 37*); the B cells must therefore stand or fall by this specificity.

Not all B cells in Ig transgenic mice are so homogeneous, however, because a certain proportion of B cells escape the negative feedback of allelic exclusion, resulting in rearrangement and expression of endogenous heavy or light chain genes, or both (*37*). The proportion of B cells falling into this category varies from only a few percent to greater than 80% (*15, 37*), and this appears to be influenced both by the age of the transgenic mouse as well as by the particular Ig-gene construct introduced and its site or mode of integration (*38*). Some of these variant B cells have entirely deleted or inactivated the transgenes, whereas others apparently either decrease expression of the transgene or simply coexpress endogenous- and transgene-encoded antibody chains (*37, 38*). Relative to B cells expressing only the transgene-encoded receptors, the variant B cells expressing endogenous antibody specificities appear to be selectively retained and expanded in peripheral lymphoid tissues of the mice, presumably because of the need to build a functional B cell repertoire. In Ig transgenic mice expressing self-reactive antibody specificities, there is an even greater tendency for such variant B cells to accumulate, since self tolerance results in strong negative selection against B cells bearing only the transgene product. In order to avoid the complications posed by the presence of these variant B cells, experiments must be carefully designed in order to include unequivocal markers to distinguish transgene-encoded antibodies from endogenous antibodies or mixed molecules.

Three basic experimental models can be used to study B cell tolerance in Ig transgenic mice. The simplest approach, in theory, entails the introduction of heavy and light chain genes encoding a self-reactive antibody into separate lines of transgenic mice (Fig. 4A). Since high-affinity antigen binding usually requires a specific combination of heavy and light chains, the individual H-chain and L-chain transgenic mice need to be mated together to produce H + L Ig transgenic offspring in which large numbers of autoreactive B cells would be generated. Ideally, however, the model must include a control group of mice, the members of which carry and express both heavy and light chain Ig transgenes but lack the relevant self antigen, so that any change in B cell frequency and function in the presence of self antigen can be reliably ascribed to self tolerance. One way to achieve this goal is to introduce the heavy and light chain genes for an antibody specific for a polymorphic self antigen, such as MHC molecules (Fig. 4B), into a mouse strain lacking the relevant *MHC* allele, and then to mate the resulting Ig transgenic mice with partners that carry the antibody-reactive form of the self antigen (*39*). An alternative but more complicated approach (Fig. 4C) involves introducing heavy and light chain genes encoding an antibody specific for a normally foreign antigen, such as hen egg lysozyme (HEL), into one line of transgenic mice, while introducing the gene for HEL itself into a separate line of transgenic mice (*15*). By mating the two types of transgenic mice, B cell tolerance can be studied in double-transgenic offspring where the anti-lysozyme B cells encounter lysozyme as a self antigen during their development in vivo, and the effects of this encounter can be assessed by comparison with Ig transgenic littermate controls lacking the lysozyme transgene. The primary advantage of the third approach is the opportunity it provides for systematically changing the expression or structure of the transgene-encoded self antigen itself by introducing modified or regulatable gene constructs (*14, 40*).

Self Tolerance in B Cells Specific for Lysozyme

In our experiments, we elected to study self tolerance to the normally foreign protein antigen, HEL. Lysozyme presents a number of advantages in that it is an extremely well-characterized antigen, it is easy to work with, and its expression in transgenic mice would be unlikely to alter the physiological or immunological milieu of the mice (*41*). In transgenic mice carrying gene constructs encoding lysozyme under transcriptional control of either the mouse metallothionein or mouse albumin promoter, lysozyme was expressed as a new self antigen and accumulated in secreted form in the serum (*14, 15*). Consequently, specific tolerance to lysozyme was induced and in contrast to nontransgenic littermates, the lysozyme-transgenic mice could no longer mount strong T or B cell responses to the antigen (*15, 16*). As described above (Fig. 2), tolerance within the B cell compartment was limited to higher affinity lysozyme-specific B cells.

Immunoglobulin transgenic mice were produced in parallel, by using rearranged heavy and light chain genes from a B cell hybridoma, HyHEL10, which secretes a structurally and functionally well-characterized antibody with high affinity (2×10^9 M^{-1}) for HEL (*30, 42*). The heavy chain transgene was constructed with a relatively large segment of the Ig H-chain μ-δ constant region locus, the aim being to ensure that the B cells could differentially express both IgM and IgD isotypes of membrane Ig and undergo maturation in a manner comparable to that of nontransgenic mice (*43*). To distinguish transgene-encoded IgM and IgD from endogenous IgM and

IgD, the heavy chain constant-region gene segment was derived from a different mouse strain, so that amino acid differences between endogenous and transgene-encoded heavy chains could be detected by binding of "anti-allotype" monoclonal antibodies.

Fluorescence activated cell sorter (FACS) analysis of spleen or lymph node cells from the resulting Ig transgenic mice showed that allelic exclusion was particularly profound in these mice, as approximately 90% of peripheral B cells expressed only transgene-encoded IgM and IgD and bound lysozyme with the same high affinity as the original hybridoma protein (*15, 40*). Moreover, even in the absence of specific immunization some transgene-expressing B cells differentiated into plasma cells, resulting in high concentrations of anti-lysozyme IgM in the serum. When, however, these Ig transgenic mice were mated with lysozyme-transgenic mice to produce double-transgenic progeny (Fig. 4C), the presence of lysozyme in the double-transgenic animals resulted in almost complete cessation of secretion of antibodies to lysozyme. Thus B cell tolerance to lysozyme, previously observed in lysozyme-transgenic mice (Fig. 2), was maintained in the double-transgenic animals despite the presence of large numbers of anti-lysozyme B cell precursors. Intriguingly, FACS analysis of spleen and lymph node cells from the double-transgenic animals revealed that the functionally silent anti-lysozyme B cells had not, in fact, been clonally deleted but persisted in similar or only slightly reduced numbers to those found in peripheral lymphoid tissue from Ig transgenic littermates (*14, 15*). On transfer into nontransgenic recipients, the tolerant B cells from the double-transgenic animals remained poorly responsive, even when stimulated with antigen and helper T cells (*14, 15*). Thus, B cell tolerance to lysozyme in double-transgenic mice appears to involve functional inactivation or clonal anergy (*9*), rather than clonal deletion of self-reactive cells.

Self Tolerance in B Cells Specific for MHC

The experimental approach taken by Nemazee and Bürki (*39*), illustrated in Fig. 4B, was to introduce rearranged light and heavy chain genes encoding the IgM form of an antibody specific for H-2K and D class I MHC antigens of the k-haplotype ($H\text{-}2^k$) into the germline of $H\text{-}2^d$ mice. In the resulting $H\text{-}2^d$ Ig-transgenic animals, the transgene-encoded anti-MHC antibody was not self-reactive and approximately 50% of peripheral B cells expressed transgene-encoded IgM, as assessed by binding of a monoclonal antibody specific for "idiotypic" determinants that were unique to the particular combination of transgene-encoded heavy and light chains. The remaining 50% or more of peripheral B cells failed to react with the anti-idiotype antibody and presumably expressed endogenous heavy or light chains, or both. In addition to large numbers of peripheral B cells expressing transgene-encoded IgM on their cell surface, high titers of antibodies to $H\text{-}2^k$ (anti–$H\text{-}2^k$ antibodies) were secreted into the serum of the $H\text{-}2^d$ animals.

When the $H\text{-}2^d$ Ig-transgenic mice were mated with nontransgenic $H\text{-}2^{k/d}$ (heterozygous) strain mice, anti–$H\text{-}2^k$ antibody secretion was completely abolished in those progeny that expressed $H\text{-}2K^k$ and D^k proteins, in the same manner as had been observed in the anti-lysozyme double-transgenic mice. However, in contrast to the lysozyme model, no B cells expressing the transgene-encoded idiotype were detected by FACS analysis of cells from peripheral lymphoid organs of $H\text{-}2^{k/d}$ Ig-transgenic mice, suggesting that B cells expressing the transgene-encoded anti-MHC specificity had been clonally deleted as a consequence of encounter with self–$H\text{-}2^k$ antigens.

The strikingly different outcome of self tolerance in the lysozyme and MHC models raises two key issues: what accounts for B cell anergy or deletion, and to what extent do these two processes involve entirely distinct cellular and molecular events? The answers to these questions are not yet known; however, some clues have been obtained by studying the link between receptor down-regulation and tolerance induction, the dependence on a threshold receptor occupancy, and the relationship between tolerance and different stages of B cell maturity.

Tolerance Is Correlated with Receptor Down-Regulation

In the lysozyme double-transgenic mice, the functionally silenced state of the anti-lysozyme B cells was accompanied by a 90 to 98% decrease in membrane IgM, but not IgD, on the cell surface of the B cells (*15*). A tight correlation between the functionally tolerant state and IgM down-regulation was observed in several other experiments. For example, a failure in B cell tolerance was found to be associated with minimal down-regulation of membrane IgM in two different variant combinations of double-transgenic mice (*40*), whereas induction of tolerance in mature B cells was accompanied by a rapid decrease in membrane IgM density (*40*). Receptor down-regulation may also be linked to tolerance induction by clonal deletion in the $H\text{-}2^k$ Ig transgenic mice, since developing B cells in

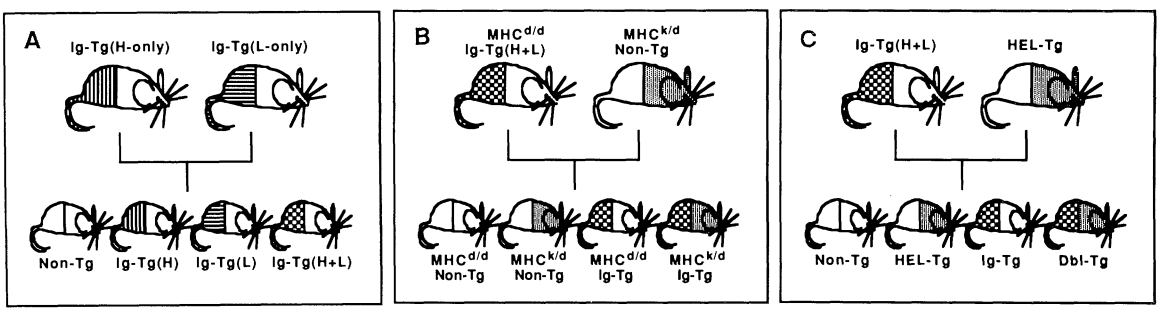

Fig. 4. Immunoglobulin transgenic models to study B cell tolerance. (**A**) In the first model, rearranged heavy and light chain genes are introduced separately, to create hemizygous transgenic lines with either the heavy chain gene or the light chain gene. The two types of mice are then mated together, so that one quarter of the offspring inherit both genes; the combination of specific heavy and light chains in these mice potentially leads to expression of high-affinity autoreactive antibodies by many of the B cells. (**B**) In the second model, heavy and light chain gene constructs are microinjected together so that the two types of transgenes become cointegrated and are inherited in subsequent generations as a single locus. It was shown by using an antibody that reacts with MHC molecules from $H\text{-}2^k$ but not $H\text{-}2^d$ strain mice, and introducing the Ig genes initially into $H\text{-}2^d$ mice followed by crossing to heterozygous $H\text{-}2^{k/d}$ strain mice, that one-quarter of the offspring will carry the Ig genes as well as the antibody-reactive self-MHC allele (*39*). (**C**) The third approach only differs from the second in that the introduced Ig genes encode an antibody specific for a transgene-encoded self antigen rather than a polymorphism in an endogenous antigen. In this example, hemizygous anti-lysozyme Ig transgenic mice are mated with hemizygous lysozyme transgenic mice, resulting in litters in which one-quarter of the offspring are "double-transgenic" (*15*).

the bone marrow of such mice, which apparently have not yet been deleted, were shown to express markedly reduced levels of membrane IgM (*39*).

The apparent link between tolerance and receptor down-regulation may provide a clue to the cellular processes involved. In the anti-MHC transgenic mice, where IgM is the only isotype of antigen receptor expressed by the B cells, it is conceivable that down-regulation of IgM may reduce the overall number of antigen receptors expressed to such an extent that the B cells can no longer home to and persist in peripheral lymphoid organs, thus accounting for their absence in these sites. Alternatively, while the B cells that persist in the lysozyme double-transgenic mice also express IgD antigen receptors, which are not down-regulated, the cells are nevertheless hyporesponsive despite the fact that IgD appears fully capable of mediating B cell activation (*44*). It is therefore possible that B cell tolerance may involve a process of antigen receptor desensitization as well as down-regulation, comparable to that described for many other cell surface receptors (*45*).

Requirement for Threshold Receptor Occupancy

Self antigen concentration and antibody affinity, as discussed previously, both appear to be important for induction of B cell tolerance in animals with a normal B cell repertoire, implying that tolerance involves binding of a threshold amount of antigen. It has been possible to test this hypothesis in the anti-lysozyme Ig transgenic mice, where the affinity of the antibody expressed by most of the B cells is uniform. Thus, when these Ig transgenic mice were mated with a number of lines of lysozyme-transgenic mice expressing different concentrations of lysozyme, B cell tolerance failed to occur in double-transgenic animals in which the free lysozyme concentration resulted in only 4.5% of the Ig receptors on the B cells being occupied with lysozyme, whereas tolerance was observed in double-transgenic mice where 45% of the receptors were occupied (*40*). The requirement for a threshold level of antigen-receptor occupancy in order to induce tolerance and receptor downregulation again has parallels with desensitization of other cell surface receptors, where downregulation and desensitization are initiated by ligand-binding in excess of a certain threshold (*45*).

A similar requirement for a threshold receptor occupancy may account for the failure to induce tolerance or receptor downregulation in double-transgenic offspring from matings between the anti-H-2K^k Ig transgenic mice and transgenic mice expressing a secreted form of the H-2K^k molecule (*46*). In such double-transgenic animals, the concentration of soluble H-2K^k (*47*) and the affinity of the anti-MHC antibody expressed on the B cells (*48*) is such that less than 11% of the receptors on the B cells may be occupied by soluble H-2K^k (*49*). It would be useful, by way of contrast, to know the level of receptor occupancy with cell surface H-2K^k in the tolerant *H-2K$^{k/d}$* Ig transgenic mice (*39*) and to ascertain whether a different threshold exists for tolerance to multivalent self antigens.

Induction of Tolerance in Mature B Cells

Hypermutation of Ig genes in mature peripheral B cells results in the generation of a "second wave" of potentially high-affinity autoreactive B cells, thus necessitating a mechanism for tolerance that can silence B cells at this stage in their differentiation. Classical models of tolerance induced by exposure to exogenous antigens have repeatedly shown that mature B cells can indeed be rendered unresponsive in vivo, although it has not been possible to follow the fate of the B cells in such models due to their low frequency (*9, 18, 50, 51*). This problem has been overcome in the anti-lysozyme Ig transgenic mice, which contain large numbers of nontolerant, mature follicular B cells specific for lysozyme (*14, 15, 52*). Thus, when mature B cells from these mice were transferred into lysozyme-transgenic recipients expressing above-threshold concentrations of lysozyme, they were also rendered tolerant (*40*). Moreover, induction of tolerance in mature B cells appeared very similar to the unresponsive state observed in the intact lysozyme double-transgenic animals, in the sense that it involved functional silencing rather than clonal deletion, required an identical threshold of receptor occupancy, and resulted in down-regulation of membrane IgM on the B cells (*40*).

The observation that mature B cells can either be activated or tolerized by encounter with foreign or self antigens, respectively, raises one of the fundamental issues of self tolerance, namely, what guides individual lymphocytes to respond in the appropriate manner? Foreign and self antigens do not appear to differ in their physical properties, which implies that some difference in the way the two kinds of antigen are encountered must be important. In the case of mature B cells, qualitative or quantitative differences in the level and duration of antigen-receptor occupancy, together with second signals derived from the B cell's particular microenvironment including those from T cells (*53*) and macrophages, may all help to ensure that the B cell makes the correct response to self and foreign antigens. Determining precisely how these different signals are integrated in vivo to give rise to regular patterns of cell behavior will be an important, and very difficult, problem to resolve.

Two Distinct Mechanisms of Self Tolerance?

The induction of self tolerance in mature peripheral B cells contrasts with an alternative mechanism of B cell tolerance originally proposed by Lederberg (*8*). In this model, self-reactive B cells were considered to be selectively eliminated as a consequence of interactions with antigen during an immature stage of B cell development, when the B cell might be intrinsically prone to respond to antigen in a negative fashion. In support of the Lederberg model, a large number of in vitro studies have demonstrated that immature B cells are much more prone to irreversible receptor downregulation and inactivation than are mature B cells (*9, 51, 54*). Similarly, recent studies of T cell tolerance suggest that antigen-encounter or receptor cross-linking on immature T cells results in tolerance, programmed cell death, and clonal deletion, whereas the same stimuli are mitogenic for mature T cells (*55*). Mature T cells can be rendered tolerant under some circumstances, but this appears to involve functional inactivation rather than deletion (*56*), much as is observed in mature peripheral B cells in the lysozyme Ig transgenic model.

From these data, it is reasonable to consider that B cell deletion in the anti–H-2K transgenic mice and B cell inactivation in the anti-lysozyme transgenic mice may reflect two distinct cellular processes. Clonal deletion may only occur if the B cells encounter self antigen early in their development within the bone marrow and may fail to be induced in the lysozyme double-transgenic mice because either lysozyme is not encountered by bone marrow B cells or lysozyme is in some way unable to initiate deletion of such cells. The former option appears to be unlikely, since lysozyme is a small secreted protein that would be expected to gain access to the extravascular microenvironment of the bone marrow, and it can, in fact, be demonstrated already bound to the receptors of immature bone marrow B cells in the double-transgenic mice (*52*). The other

alternative, namely, that binding of lysozyme is sufficient to induce functional inactivation but not clonal deletion, has considerable merit since, in contrast to cell surface H-2K^k, lysozyme is a predominantly monovalent antigen and would not extensively cross-link membrane Ig on the surface of immature B cells. A requirement for extensive receptor cross-linking to induce deletion of immature B cells but not to induce functional inactivation of mature B cells could well explain the long-standing experimental paradox that exogenous multivalent antigens are uniquely able to induce tolerance in immature B cells in vitro, whereas exogenous monovalent antigens are as good if not better at inducing tolerance in mature peripheral B cells in vivo (*9, 17, 18, 50, 51, 54*).

Because the B cell repertoire is generated in two phases (Fig. 3), the existence of two mechanisms of B cell tolerance could make good physiological sense. A central mechanism of tolerance that deletes immature B cells specific for multivalent self antigens may be necessary despite the predominantly low affinity of such cells, since even low-affinity autoantibodies can bind with high avidity to multivalent self antigens. A second mechanism acting on peripheral B cells, which bind any type of self antigen above a critical threshold, such as functional inactivation, will be essential to censor potentially pathogenic autoreactive B cells that have either dangerously increased their affinity for self antigen or only acquired self-reactivity as a consequence of hypermutation. One way to confirm the validity of this concept of distinct central and peripheral B cell tolerance mechanisms is to make further use of the potential of transgenic models, by selectively altering either the structure and expression of self antigens or the isotype of the B cell's receptor through the introduction of modified gene constructs. Ultimately, an understanding of the molecular and cellular events involved in clonal inactivation or clonal deletion should resolve the extent to which the two processes are related, how they differ from antigen-induced activation, and how they come to fail in clinical and experimentally induced autoimmunity.

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