# Induction of Self-Tolerance in T Cells But Not B Cells of Transgenic Mice Expressing Little Self Antigen

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**Self-tolerance to a transgene-encoded protein, hen egg lysozyme, was examined in the T and B cell repertoires of a series of lines of transgenic mice that expressed different serum concentrations of soluble lysozyme. T cells were tolerant in all lines in which lysozyme was expressed irrespective of the antigen concentration, whereas B cell tolerance did not occur when the serum lysozyme concentration was less than 1.5 nanograms per milliliter (0.1 nM). Induction of elevated transgene expression could restore B cell tolerance. These findings support the hypothesis that autoimmune disease may in some instances arise through a bypass of T cell tolerance.**

IN CONTRAST TO THE ACQUISITION OF immunity, which depends on selective expansion of T and B lymphocytes specific for foreign antigens, self-tolerance appears to be achieved predominantly by an inverse process involving elimination or inactivation of self-reactive T and B cells (*1–3*). The factors that determine these opposite responses to self and foreign antigens, however, remain unclear. Induction of unresponsiveness to foreign antigens administered in such a way as to mimic acquisition of tolerance to bona fide self antigens, has established relationships between unresponsiveness and differences in the structure or dose of antigen as well as in the route and timing of its administration (*4*). It has nevertheless been difficult to exclude the possibility that some of these variables might be involved in the control of immune responses to foreign antigens, rather than in the process of tolerance to bona fide self antigens. Comparisons have also been made between the immune repertoires of genetically related animals distinguished only by the presence or absence of particular antigenic determinants (*5*). Although this approach has indicated the presence or absence of T or B cell tolerance to particular self antigens, it has been difficult to reconcile conflicting results obtained with different self antigens because of the inability to vary the expression or structure of any one self antigen.

The advent of transgenic technology has provided a strategy with the potential to resolve such problems (*6*). Not only are transgene-encoded proteins likely to be expressed and presented in an identical manner to self antigens (in contrast to exogenously administered antigens), but the concentration, structure, and site of production of a single self antigen can be manipulated by engineering systemic changes in the introduced gene constructs. Using this approach, we generated a series of transgenic mouse lines on an inbred C57BL/6 background by introducing gene constructs containing the coding regions of the hen egg lysozyme (HEL) gene linked to 5′ promoter elements derived from either the zinc-inducible mouse metallothionein-I gene or the liver-specific mouse albumin gene (*3, 7*). In metallothionein-lysozyme (ML-series) and albumin-lysozyme (AL-series) transgenic mice, transgene-encoded lysozyme was constitutively expressed and accumulated as a soluble serum protein at concentrations that were characteristic for a particular line (Fig. 1), ranging from a mean HEL concentration of 58 ng/ml (line AL3) to less than 0.5 ng/ml (lines ML4 and ML3a).

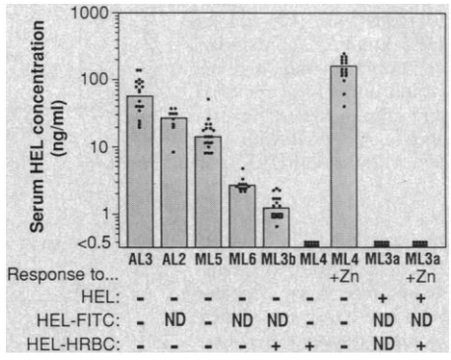


**Fig. 1.** Lysozyme expression and summary of immune responses to lysozyme in different transgenic mouse lines. Lysozyme concentrations were determined by enzyme-linked immunosorbent assay (ELISA) (*7*) in the sera of individual transgenic animals from each line, as indicated by dots. Columns represent geometric means. Where indicated, transgenic mice from lines ML3a and ML4 were given drinking water containing 25 mM zinc sulfate for a minimum of 5 days before assay. Line ML3a does not express lysozyme. The results of testing for tolerance to lysozyme are summarized from data presented in Figs. 2, 3, and 4, and from measurement of serum antibody responses to HEL and HEL-HRBC in all lines by ELISA assay of lysozyme-binding immunoglobulin G (IgG) (*3*). ND, not determined.

One of the highest expressing lines, ML5, is specifically tolerant to lysozyme within both the T and B cell repertoires (*3*). To examine the effects of different antigen concentrations in the serum on induction of tolerance, we carried out similar analyses on the other lines (Fig. 1). Initially, (C57BL/6 × CBA)$F_1$ transgenic mice and nontransgenic littermates from each line were tested for their ability to produce lysozyme-binding antibodies when injected with lysozyme in complete Freund's adjuvant (CFA) (Fig. 1 and Fig. 2, A to D). With the exception of ML3a, transgenic mice from each of the lines made little or no antibody response to lysozyme as compared with their nontransgenic littermates, indicating that profound tolerance was present even in transgenic mice with low amounts of expressed antigen, such as ML4. The only nontolerant line, ML3a, appeared not to express the transgene, since lysozyme remained undetectable in these mice even after induction of the metallothionein promoter, but it did serve as an additional control to the nontransgenic littermates used in the experiments described below (Fig. 1).

The failure to produce antibodies to lysozyme in each of the tolerant lines could have been due to tolerance within the T or B cell repertoires, or both. Two approaches were used to test for tolerance to lysozyme selectively within the T cell repertoire: a hapten-carrier system and T cell proliferation. In the first, (C57BL/6 × CBA)$F_1$ mice were immunized with lysozyme coupled to the foreign hapten, fluorescein isothiocyanate (FITC). Antibody production to the FITC determinant therefore depended on interaction of FITC-specific B cells with carrier (lysozyme)–specific helper T cells. In contrast to nontransgenic littermates, little or no FITC-binding antibody was produced by transgenic animals from each of the tolerant transgenic lines (Fig. 2, E to H), indicating that profound tolerance had developed within the helper T cell compartment even at very low amounts of self antigen expression. Similar results were obtained by measuring the proliferative response of lymph node cells from $F_1$ transgenic and nontransgenic donors to lysozyme in vitro (Fig. 3).

A reverse approach was used to test for tolerance within the B cell repertoire by immunizing with lysozyme, acting as a hapten, conjugated to the foreign carrier, horse red blood cells (HRBC). To ensure the

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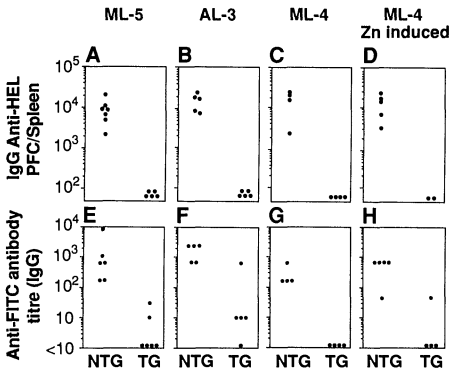


**Fig. 2.** Tolerance within the helper T cell repertoire of transgenic mice from different lysozyme-transgenic lines. (**A** to **D**) Tolerance to lysozyme was initially determined by immunizing (C57BL/6 × CBA)$F_1$ transgenic mice (TG) and nontransgenic littermates (NTG) intraperitoneally (IP) with 100 μg of HEL emulsified in CFA. The animals were boosted 80 days later with 10 μg of soluble HEL IP, and plaque-forming cells (PFC) secreting lysozyme-binding IgG were enumerated in the spleen 5 days after the boost, as described previously (*3*). (**E** to **H**) Helper T cell responses to lysozyme were assayed by immunizing $F_1$ animals with 100 μg of HEL-FITC (*17*) in CFA, and measuring FITC-binding antibodies in the serum 35 days later by ELISA (*17*).

absence of a significant helper T cell response to the lysozyme moiety of the conjugate, experiments were performed in C57BL/6 mice, thus taking advantage of their low responder status to HEL, which is associated with the class II genes of the major histocompatibility complex (MHC) (*8*). In secondary responses to HEL-HRBC, a 50 to 90% reduction was observed in the numbers of cells secreting lysozyme-binding antibody from the spleens of transgenic mice from lines AL3 and ML5 (Fig. 4, A and B). More importantly, the median affinity of the residual lysozyme-specific antibody-secreting cells in the transgenic mice was decreased by 95 to 99% relative to that of nontransgenic littermates (Fig. 4, F and G), indicating that there was a drastic reduction in the number of cells secreting high-affinity antibody, despite the relatively small decrease in the overall number of antibody-secreting cells detected in some cases (Fig. 4B). In contrast, lines ML3b and ML4, which express much lower concentrations of serum lysozyme, displayed no difference between transgenic mice and nontransgenic littermates either in the numbers of antibody-secreting cells (Fig. 4, C and D) or in their median affinity (Fig. 4, H and I), despite the existence of tolerant T cells in the transgenic animals. Thus, high-affinity self-reactive B cells could still develop and respond normally in animals with less than $10^{-10}$ M serum lysozyme, provided an alternative source of T cell help was available.

Our next experiments were to confirm the relationship between the concentration of antigen expressed and acquisition of self-tolerance within the high-affinity component of the B cell repertoire. Adult mice from the ML4 transgenic line, which normally failed to exhibit B cell tolerance, were given drinking water containing 25 mM zinc sulfate for 2 weeks to enhance transcription of the metallothionein-lysozyme gene construct and elevate the serum concentration of lysozyme to $10^{-8}$ to $10^{-9}$ M (Fig. 1). After immunization with HEL-HRBC, the zinc-induced, in contrast to the uninduced, ML4 transgenic mice showed a marked decrease in both the number and affinity of responding lysozyme-specific B cells compared with nontransgenic littermate controls (Fig. 4, D, E, I, and J).

Our data on the selective failure of self-tolerance within the B cell, but not in the T cell, repertoires of transgenic mice expressing less than $10^{-10}$ M serum lysozyme confirm that the differential sensitivity of T and B cells to tolerance induction, previously observed following induction of unresponsiveness to exogenous antigens (*9*), also applies to naturally acquired tolerance to self antigens. The phenomenon of differential sensitivity may be due to differences in antigen recognition between the two classes of lymphocyte. Antigen recognition by T cells involves cell-cell contact and the presentation of peptide fragments in association with MHC molecules by specialized antigen-presenting cells (*10*), which could increase the sensitivity of self antigen detection. In contrast, antigen recognition by B cells involves direct antigen-antibody interaction, and therefore may be more directly related to free antigen concentration (*4*), as we have observed previously in antibody transgenic mice (*11*). The present observation that tolerance is only induced in higher affinity B cells and only when lysozyme is expressed at greater than $10^{-10}$ M indicates the need for a similar if not identical threshold for induction of self-tolerance in the normal B cell repertoire.

The importance of self antigen concentration as well as receptor affinity in determining the outcome of interactions between B cells and self antigen is illustrated by the common occurrence in otherwise healthy individuals of natural autoantibodies. Many of these antibodies recognize self antigens present above the nominal threshold of

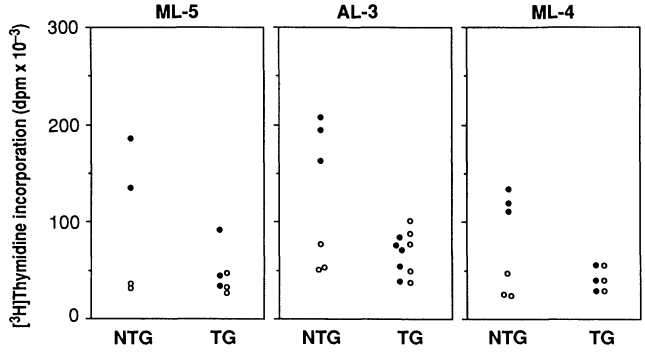


**Fig. 3.** Tolerance within the T cell repertoire of lysozyme transgenic mice from lines with the highest, intermediate, and lowest serum concentrations of HEL. Transgenic (TG) and nontransgenic (NTG) animals were immunized in the hind footpads with 100 μg of HEL in CFA. Ten days later, popliteal and inguinal lymph nodes were removed and the proliferative response measured with (●) and without (○) HEL, as described (*8*).

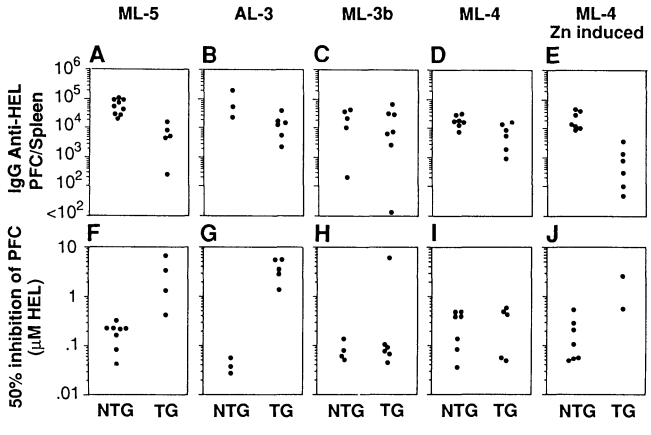


**Fig. 4.** Tolerance within the B cell repertoire of transgenic mice from different lysozyme-transgenic lines. Lysozyme-specific B cell responses were elicited by immunizing C57BL/6 transgenic mice (TG) and nontransgenic littermates (NTG) with HEL conjugated to HRBC ($2 \times 10^8$ per mouse), to bypass T cell tolerance, and the animals were boosted with the same dose 2 weeks later. (**A** to **E**) The numbers of PFC secreting HEL-specific IgG were determined 5 days after the boost (*3*). (**F** to **J**) To estimate the number of high-affinity antibody-secreting cells in the responding innoculum, we estimated the relative median affinity of the antibody-secreting cells in parallel by determining the concentration of soluble lysozyme required to inhibit development of 50% of the plaques (*3*). In some transgenic animals, very low absolute numbers of PFC made it impossible to derive an affinity measurement.

$10^{-10}$ M (*12*), but, being derived from nonmutated germ-line immunoglobulin genes (*13*), they are predominantly of low affinity (*12*) and are nonpathogenic. Alternatively, when soluble self antigens are expressed at subthreshold concentrations, the failure to induce tolerance in high-affinity self-reactive B cells creates the potential for production of pathogenic autoantibodies of high affinity should T cell tolerance be bypassed (*7, 14*). Although most self antigens are present at amounts above this threshold, such a situation may explain why it has been relatively easy to raise high-affinity autoantibodies against certain autologous (*15*) or transgene-encoded (*16*) antigens in experimental models. Moreover, the nominal threshold of self antigen required for inducing B cell tolerance within the high-affinity component of the B cell repertoire may not be absolute but may be influenced by variables such as the tissue site of expression and the valency of individual antigens. Collectively, these findings suggest that the selective failure of B cell tolerance to particular self antigens may provide one route toward the development of clinical autoimmune disease, particularly under conditions where B cells are chronically stimulated either nonspecifically or by foreign antigens that cross-react or form complexes with self antigens (*14*).


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17. FITC-HEL was prepared with three-times recrystallized HEL (Sigma) that was further purified by ion-exchange chromatography. Fluorescein isothiocyanate (5 μl of 5 mg/ml in dimethyl sulfoxide) was added to HEL (5 mg/ml) in 1 ml of 0.1 M $NaHPO_4$, pH 10.0, and incubated for 30 min at 37°C. The reaction products were separated by chromatography over Sephadex G-25, resulting in a hapten:protein ratio of 0.8. FITC-binding antibodies were measured by enzyme-linked immunosorbent assay (ELISA) (*3*), except that the microtiter plates were coated with FITC-conjugated bovine serum albumin (2 μg/ml) (hapten:protein ratio of 6). Titers were determined from the last dilution with an optical density (OD) greater than twice the mean OD for preimmune sera.


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# Peripheral Selection of the T Cell Repertoire


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**T lymphocytes undergo selection events not only in the thymus, but also after they leave the thymus and reside in the periphery. Peripheral selection was found to be dependent on T cell receptor (TCR)–ligand interactions but to differ from thymic selection with regard to specificity and mechanism. Unlike thymic selection, peripheral selection required binding of antigen to the TCR, and it induced expansion of T cell clones. Tolerance to self antigens that are restricted to the periphery occurred through the elimination of self-reactive T cells and by the clonal anergy, which was associated with down-regulation of the αβ TCR and CD8.**


DEVELOPMENT OF T CELLS TAKES place in the thymus, where the TCR repertoire is generated by random gene rearrangement and pairing of TCR α and β chains. Potentially autoaggressive thymocytes are removed from this repertoire by "negative selection" (*1, 2*). Maturation of thymocytes requires "positive selection," which involves binding of the TCR to major histocompatibility complex (MHC) molecules (*3*): TCR interactions with class I MHC molecules direct $CD4^+$ $CD8^+$ thymocytes into the $CD8^+$ lineage, whereas interactions with class II MHC molecules result in maturation into $CD4^+$ cells (*4–7*).

Thymic selection may not be the sole mechanism by which the T cell repertoire is formed. Selection of T cell specificities may also occur in the periphery, to which the expression of certain self antigens may be limited. Tolerance to these antigens would require an extrathymic mechanism (*8*). In addition, peripheral selection events may expand or "suppress" certain clones. These selection events may be of particular importance in the normal adult mouse, in which the maintenance of the peripheral T cell compartment is largely independent of thymic output and the majority of T cells are generated by peripheral expansion (*9–14*): once the peripheral pool of T cells is seeded from the thymus early after birth, it may be sustained throughout life independently of further cell export from the thymus (*14*). Extrathymic expansion of T cells is not necessarily a response to exogenous antigens because it does not require intentional antigenic stimulation and it occurs in germ-free mice (*12, 13*).

To determine whether TCR-ligand interactions are required for peripheral expansion, we analyzed the in vivo expansion of αβ TCR transgenic T cells with receptors of known specificity. The genes for the α and β chains of a TCR that recognizes the male antigen HY when presented by the H-$2D^b$ class I MHC molecule were introduced into mice, which were then backcrossed to the C57Bl/6 (B6) background. In these mice, endogenous $V_\beta$ gene segments do not rearrange, and the transgenic β chain is expressed on all T cells (*2*). Not all cells express the α transgene (*3*). Because specificity for HY requires the transgenic α and β chains ($\alpha_T$ and $\beta_T$) as well as CD8 molecules (*15*), male antigen–specific cells are $CD8^+$ with high levels of expression of $\alpha_T$ ($CD8^+\alpha_T$ cells). Because positive selection occurs in the absence of the HY antigen, $CD8^+\alpha_T$


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