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Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice

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Immunological tolerance has been demonstrated in double-transgenic mice expressing the genes for a neo-self antigen, hen egg lysozyme, and a high affinity anti-lysozyme antibody. The majority of anti-lysozyme B-cells did not undergo clonal deletion, but were no longer able to secrete anti-lysozyme antibody and displayed markedly reduced levels of surface IgM while continuing to express high levels of surface IgD. These findings indicate that self tolerance may result from mechanisms other than clonal deletion, and are consistent with the hypothesis that IgD may have a unique role in B-cell tolerance.

Autoimmunity is a rare event despite the genetic potential of every individual to mount immune responses to autologous antigens. This is due to the fact that the immune system somehow 'learns' to recognize these antigens as self and to tolerate them. The way in which self tolerance occurs is thought to depend on a range of factors including the time of exposure to antigen during ontogeny, the tissue site of expression, concentration, and the structure of the antigen, although the relative importance of each of these factors in induction of tolerance to individual antigens remains obscure. It has been postulated that self-reactive lymphocytes, which might otherwise cause responses, either undergo clonal deletion from the pre-immune repertoire or remain in a functionally silent form as a result of early or repeated exposure to antigen (clonal anergy), contact with antigen-specific suppressor T cells or regulatory elements of an idiotypic network. These mechanisms are not mutually exclusive; for example, clonal deletion within the thymus or bone marrow could be reinforced by antigen-specific suppression in secondary lymphoid tissue.

The continuing controversy surrounding the mechanism of self tolerance stems largely from the difficulty in determining the fate of anti-self lymphocytes which, like lymphocytes with specificity for foreign antigens, are generated at very low frequencies in the pre-immune repertoire. This applies particularly to the high affinity cells believed to be important for pathogenic autoimmune responses. Thus although self-reactive T cells and B cells have been detected in secondary lymphoid tissue, their functional significance remains unclear for two reasons. First, their detection has relied on the use of complicated functional assays or read-out systems that pick up the more frequent lymphocytes with very low affinity for antigen; secondly the cells represent an unknown fraction of the total output of anti-self lymphocytes from thymus or bone marrow.

One elegant approach to following the fate of self-reactive lymphocytes has involved the identification of naturally occurring situations in which the frequency of T cells specific for certain polymorphic cellular antigens is relatively high and can be measured directly by anti-Vγ monoclonal antibodies. In such situations the presence of self antigen clearly led to clonal deletion of 70–97% of anti-self T cells. However, this strategy is difficult to extend to other T cell specificities where there is less correlation between V-region usage and antigen specificity and where the frequency of anti-self T cells is lower; nor can it be readily applied to B cells where the V-region repertoire is much larger.

An alternative strategy is to generate high frequencies of anti-self lymphocytes in the pre-immune repertoire, through the introduction of rearranged T-cell receptor or immunoglobulin genes into the germline of transgenic mice. Here, we have followed this approach by generating two kinds of transgenic mice. The first kind carries the gene for a normally foreign protein, hen egg lysozyme (HEL), expressed in such a way that the transgenic antigen is recognized as self and leads to tolerance in anti-lysozyme T and B cells. The second kind of transgenic mouse carries rearranged immunoglobulin heavy and light chain genes encoding a high-affinity anti-lysozyme antibody, resulting in high frequencies of B-cells expressing this single receptor specificity. By mating the two kinds of transgenic mice, 'double-transgenic' offspring carrying lysozyme and immunoglobulin transgenes were produced. The anti-lysozyme B cells in these double-transgenic mice were functionally silenced, but not through clonal deletion. Rather they no longer responded efficiently even after adoptive transfer to mice not previously exposed to HEL and, intriguingly, were found to display greatly reduced levels of cell surface IgM but unchanged levels of IgD. This experimental strategy has the dual advantage of providing an in vivo system of tolerance induction wherein antigenic variables can be rigorously controlled and the fate of self-reactive high-affinity B cells can be followed with precision.

Lysozyme-transgenic mice

Hen-egg lysozyme was selected as the transgenic self antigen for the following reasons: first, the biochemistry and antigenic structure of lysozyme are well defined; second, DNA clones for lysozyme and large amounts of protein were available; finally lysozyme is non-toxic in vivo and is therefore unlikely to exert non-specific effects on the immunological milieu of recipient mice. A genomic fragment containing the coding exons of the chicken lysozyme gene was linked to the mouse metallothionein I promoter (Fig. 1a). The choice of the metallothionein promoter was based on the fact that it is transcriptionally active during embryonic, fetal and adult life, and can be induced to higher levels of transcription by heavy metals such as zinc at any of these stages. The hybrid gene was microinjected into fertilised, inbred C57BL/6 eggs. Eight of 41 mice (20%) carried the transgene, and five of the eight (63%) had measurable amounts of lysozyme in their serum (0.8–28 ng ml−1, without induction by zinc).
Tolerance in lysozyme-transgenic mice

To assay for tolerance to lysozyme in the metallothionein/lysozyme transgenic mice we selected one line, ML-5, with the highest basal levels of serum lysozyme (for ML-5 offspring: median = 1.3 mg ml⁻¹, interquartile range 1.3–20 mg ml⁻¹) and a single integrated copy of the transgene. It was not, however, possible to test the ML-5 mice for tolerance by direct challenge with lysozyme since C57BL/6 mice respond poorly to this antigen due to a recessive Iγ gene effect25. C57BL/6 ML-5 mice which were hemizygous for the transgene were therefore mated with CBA (dominant high responder) mice, yielding litters of F1 animals (responders) containing equal numbers of transgenic individuals and nontransgenic controls.

As shown in Fig. 2a, little or no anti-lysozyme antibody was detected by ELISA in the sera of transgenic mice from (CBA × B6)F₁, litters immunized with lysozyme in adjuvant (HEL/CFA). By contrast, high titres were obtained in all of the nontransgenic littersmates. In theory the absence of detectable antibody in the transgenic mice could have been due to masking of the antibody by circulating lysozyme rather than tolerance, even though the low (nanomolar) serum lysozyme concentration in ML-5 mice is two orders of magnitude below that required to inhibit binding of anti-lysozyme antibodies in ELISA. To exclude this possibility formally, the number of cells actively secreting anti-lysozyme antibody was measured by plaque-forming cell (PFC) assay using lysozyme-coupled red blood cells (Fig. 2b). No direct (IgM) or indirect (IgG) plaque-forming cells were detected in any of the transgenic mice, whereas large numbers of IgG anti-lysozyme secreting cells were present in all of the nontransgenic littersmates. Thus transgenic mice from the ML-5 line are indeed tolerant to lysozyme.

The lack of an anti-lysozyme antibody response in ML-5 mice could involve inactivation of lysozyme-specific T cells, B cells, or both. Tolerance within the T-cell compartment was examined in an in vitro proliferative assay. Lysozyme-primed lymph node cells were obtained from (CBA × B6)F₁ offspring of the ML-5 transgenic line and the proliferative response was measured following restimulation with soluble lysozyme in vitro. Cells from the nontransgenic donors proliferated strongly after exposure to lysozyme as expected, whereas the response from cells of transgenic littersmates was minimal (Fig. 2c). In addition the ‘transgenic’ T cells acted as a poor source of help in an adoptive anti-hapten antibody response (data not shown).

These results showed that tolerance in ML-5 mice affects lysozyme-specific T cells, but did not indicate whether the B-cell compartment was unresponsive as well. To resolve this issue, C57BL/6 rather than F₁ offspring from the ML-5 line were immunized with lysozyme coupled to sheep red blood cells (HEL/SRBC), the latter acting as a foreign carrier to provide an alternative source of T-cell help. When the anti-lysozyme antibody response was measured by ELISA a 50-fold reduction in specific antibody titre was observed in the transgenic mice compared with their nontransgenic littersmates (Fig. 2d). In contrast, there was no difference in the anti-SRBC titres between the same two groups of mice (data not shown). Comparisons of the number of anti-lysozyme plaque-forming cells in the spleens of these mice showed a similar but less dramatic trend, nevertheless confirming an effect of tolerance on the B-cell compartment (Fig. 2e). As tolerance is thought to affect high-affinity cells preferentially2,23, the most plausible explanation for the discrepancy between the two assays was that the plaque assay detected more low affinity antibodies than the ELISA.

This possibility was confirmed by demonstrating a requirement for higher concentrations of soluble lysozyme to inhibit PFC lysis from transgenic donors than from their nontransgenic littersmates (Fig. 2f).

Immunoglobulin-transgenic mice

The findings in ML-5 mice confirmed the feasibility of inducing tolerance to transgene-encoded antigens2–27. In addition they provided further support for the concept that tolerance can influence the B-cell as well as the T-cell compartment and that this effect is most noticeable in the higher affinity B cells2. Owing to their low frequency, however, it was not possible to determine the fate of these cells nor to distinguish between the possible mechanisms of tolerance operating, such as clonal deletion, clonal anergy or suppression. To overcome this problem we...
increased the frequency of high affinity anti-lysozyme B cells by introducing rearranged immunoglobulin genes into a parallel set of transgenic mice. The hybridoma HyHEL10 (ref. 28) was selected as the source of lysozyme-specific heavy and light chain genes because the antibody secreted by this hybridoma has a high affinity for lysozyme ($K_D = 2 \times 10^{-8} M^{-1}$; M. E. Denton and H. A. Sheraga, personal communication) and the interaction of the antibody with its epitope has been characterized in detail (ref. 28 and E. Padlan, E. Silverton, S. Sheriff, G. Cohen, S. Smith-Gill and D. Davies, unpublished results). Genomic clones encoding the HyHEL10 heavy and light chains (T. B. Lavoie et al., manuscript in preparation) were used for the immunoglobulin transgene constructs. The $\gamma_1$-constant region of the HyHEL10 heavy chain gene was replaced by a segment containing the $\mu$ and $\delta$ constant region genes (Fig. 1b). The rationale for using the complete $\mu-\delta$ locus was to ensure that the progression from expression of IgM alone to co-expression of IgM plus IgD which is observed during normal B-cell maturation, could occur in B cells expressing the transgene. The $\mu$ and $\delta$ genes were derived from BALB/c (IgH allotype) mice. This allowed transgene-derived heavy chains to be distinguished by anti-allyotypic monoclonal antibodies from products of the endogenous C57BL/6 (IgH$^b$ allotype) heavy chain genes.

The light and heavy chain genes were co-injected into C57BL/6 eggs, as co-injection usually results in the integration of both genes into a single chromosomal locus and co-segregation during breeding. Seven of 47 mice born were transgenic (15%); six of these carried copies of both light and heavy chain transgenes whereas the seventh contained only the heavy chain. High serum titres of anti-lysozyme antibodies were detected in all six animals with both immunoglobulin genes. The heavy chain-only founder had very low levels of anti-lysozyme activity but high levels of a-allyotype IgM (data not shown). These data confirm the importance of both transgene-derived chains for high-affinity lysozyme binding.

One line, MD-3, was bred from a male immunoglobulin (Ig)-transgenic founder which carried a single integration site containing multiple copies of heavy and light chain transgenes. To determine the frequency of anti-lysozyme B-cells in MD-3 offspring, spleen cells were stained with monoclonal anti-allyotype antibodies or a sandwich of soluble lysozyme followed by a noncompeting monoclonal antibody to lysozyme. Two-colour FACS analysis showed that over 90% of the splenic B-cells expressed transgene-derived a-allyotype heavy chains as IgM and/or IgD (Fig. 3b), and that 60-90% of these cells bound lysozyme (Fig. 3c and Table 1). Furthermore, only 1-6% of spleen cells bearing the endogenous b-allyotype IgM or IgD were
Fig. 3  Two-colour FACS analyses of spleen cells from non-transgenic, Ig-transgenic and double-transgenic mice. Spleen cells were stained with monoclonal antibodies specific for IgM and IgD of the b-allotype to identify cells carrying endogenous C57BL/6 heavy chains (a), or monoclonal antibodies specific for IgM and IgD of the a-allotype to identify cells bearing transgene-encoded IgM or IgD (b). To identify lysozyme-binding B cells (c), cells were stained with an excess of unlabelled lysozyme followed by a complementary biotinylated anti-lysozyme monoclonal antibody, and counterstained with a rat monoclonal antibody to all mouse IgM. Contour maps show correlated fluorescence in arbitrary relative fluorescence units, with fluorescein (FITC) emission on the x-axis and phycoerythrin (PE) emission on the y-axis and contour intervals at 5, 10, 20, 40 and 80 counts per square of a 4×4 channel grid.

Methods. Spleen cell suspensions from 6-week-old transgenic (MD-3.2, MD-3.6) or nontransgenic mice were stained31,32 with the following monoclonal antibodies: anti-IgM, AFS-78.25/FITC; anti-IgD, AF6-122.2/biotin; anti-IgM, DS-1/FITC; anti-IgD, AMS-15.1—biotin; anti-IgM, 331.12/FITC. For lysozyme binding, cells were stained with 200 ng ml⁻¹ HEL followed by 200 ng ml⁻¹ HyHEL9/biotin59. Binding of biotinylated reagents was revealed with streptavidin/PE (Becton-Dickinson). Four-parameter data were collected for 3×10⁶ cells on a FACS 440 with linear amplification of forward and side-scatter and logarithmic amplification of fluorescence emission. Each profile represents fluorescence data from list mode files after gating out dead cells, granulocytes and macrophages on the basis of forward and side-scatter. The percentage positively staining cells was determined from cutoffs set at x = 1.15, y = 10.0 (a and b), or at x = 1.15, y = 3.8 (c).

Table 1  B-cell frequency and phenotype in nontransgenic, Ig-transgenic and double-transgenic mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Sex</th>
<th>Age (weeks)</th>
<th>Transgene</th>
<th>HEL⁺ cells</th>
<th>Relative IgM fluorescence</th>
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<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td>HEL Ig</td>
<td>% IgM⁺⁺⁺⁺ and/or IgD⁺⁺⁺⁺</td>
<td>% HEL⁺ cells Relative IgM fluorescence</td>
</tr>
<tr>
<td>Non-Tg</td>
<td>M</td>
<td>6</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MD-3.2</td>
<td>M</td>
<td>6</td>
<td>+</td>
<td>3.7</td>
<td>16.1 9.3 62 0.35</td>
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<tr>
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<td>6</td>
<td>+</td>
<td>4.4</td>
<td>17.8 11.7 56 0.33</td>
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<tr>
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<td>6</td>
<td>+</td>
<td>5.8</td>
<td>34.2 13.3 23 0.31</td>
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<tr>
<td>MD-3.9</td>
<td>F</td>
<td>6</td>
<td>+</td>
<td>4.7</td>
<td>25.0 10.8 2.0 0.32</td>
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<tr>
<td>Non-Tg</td>
<td>M</td>
<td>8</td>
<td>—</td>
<td>48.3</td>
<td>0.2 0.42 — 0.28 3.5</td>
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<tr>
<td>MD-3.10</td>
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<td>8</td>
<td>+</td>
<td>1.2</td>
<td>7.0 13.3 39 0.30</td>
</tr>
<tr>
<td>MD-3.11</td>
<td>M</td>
<td>8</td>
<td>+</td>
<td>1.0</td>
<td>2.8 6.7 1.6 0.28</td>
</tr>
<tr>
<td>Lymph node</td>
<td></td>
<td></td>
<td>HEL Ig</td>
<td>% IgM⁺⁺⁺⁺ and/or IgD⁺⁺⁺⁺</td>
<td>% no. (×10⁶) HEL⁺ cells Relative IgM fluorescence</td>
</tr>
<tr>
<td>Non-Tg</td>
<td>M</td>
<td>8</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MD-3.10</td>
<td>M</td>
<td>8</td>
<td>+</td>
<td>1.3</td>
<td>21.5 22 0.40</td>
</tr>
<tr>
<td>MD-3.11</td>
<td>M</td>
<td>8</td>
<td>+</td>
<td>0.7</td>
<td>6.9 1.1 0.32</td>
</tr>
<tr>
<td>Bone marrow</td>
<td></td>
<td></td>
<td>HEL Ig</td>
<td>% IgM⁺⁺⁺⁺ and/or IgD⁺⁺⁺⁺</td>
<td>% no. (×10⁶) HEL⁺ cells Relative IgM fluorescence</td>
</tr>
<tr>
<td>Non-Tg</td>
<td>M</td>
<td>8</td>
<td>—</td>
<td>9.2</td>
<td>0.2 — 0.30</td>
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<tr>
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<td>+</td>
<td>ND 3.6</td>
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<tr>
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<td>8</td>
<td>+</td>
<td>ND 2.9</td>
<td>1.8 1.6 0.28</td>
</tr>
</tbody>
</table>

Transgenic littermates were genotyped as described in Fig. 4. Spleen, mesenteric lymph node, and bone marrow cell suspensions were stained and analysed by two-colour FACS as described in Fig. 3. The total number of lysozyme-binding cells (HEL⁺) = % positive × number of cells per spleen. Relative IgM fluorescence was determined in arbitrary relative fluorescence units from the peak (modal) green fluorescence (331.12/FITC) within the lysozyme-binding (HEL⁺) and non-binding (HEL⁻) populations. Where two peaks were present, as in the HEL⁺ cells from nontransgenic spleen, the values for both peaks are shown. FACS analysis of spleen cells from mice carrying only the lysozyme-transgene gave identical results to nontransgenic C57BL/6 mice. ND, not determined.
detected (Fig. 3a and Table 1) and these showed very weak staining. The low frequency of B-cells bearing endogenous heavy chains most likely reflects allelic exclusion of endogenous gene rearrangement by the already rearranged transgenes, however the degree of inhibition in MD-3 mice is far greater than has been previously observed.

Compared with the marked abnormalities reported previously in B cells from μ-transgenic mice, the B cells in MD-3 mice proved to be relatively normal by several criteria. Thus there was only a moderate reduction in their frequency and numbers in spleen and lymph nodes, although the reduction may become more marked with age (Table 1). Second, they lacked Ly-1 and Mac-1 but did express B220, J11D, and Igα in amounts identical to the predominant population of splenic B cells from the nontransgenic controls (data not shown). Finally their Ig phenotype, IgM, IgD (Fig. 3b), although different from the IgM, IgD phenotype characteristic of splenic B cells from nontransgenic adult mice, does have a normal counterpart in that it is very similar to that of B-cells in the spleen of 1-2-week-old mice.

Tolerance in double-transgenic mice

Double-transgenic mice bearing the immunoglobulin transgenes and the lysozyme transgene were produced by mating the MD-3 founder male with ML-5 females which were hemizygous for the lysozyme transgene. In the first two litters, 4/17 mice carried both antibody and lysozyme transgenes (double-transgenic), 6/17 carried only the immunoglobulin transgenes (Ig-transgenic), 6/17 carried only the lysozyme transgene (HEL-transgenic) and 1/17 was nontransgenic.

Spontaneous secretion of high serum titres of anti-lysozyme antibody, at levels comparable to those in the founder animals, was observed in the Ig-transgenic mice (Fig. 4a). By contrast, in the double-transgenic littermates, lysozyme-binding antibody was either undetectable or present in extremely low concentrations. When the serum concentration of all IgM antibodies bearing the transgenic a-allotope was measured in the double-transgenic mice (Fig. 4b) a similar although less marked reduction in titres was evident. Furthermore, the spleens of Ig-transgenic mice contained large numbers of anti-lysozyme plaque-forming cells (Fig. 4c) whereas none were detected in the spleens of double-transgenic mice, once again arguing against simple masking of the antibody by circulating lysozyme.

Two-colour FACS analysis was used to determine whether the lack of anti-lysozyme antibody secretion in double-transgenic mice was due to clonal deletion of B-cells expressing the transgenic antibody. Lymphocytes expressing transgene-encoded receptors were identified either by binding of monoclonal a-allotope antibodies specific for transgenic IgM or IgD heavy chains (IgH\(^{+}\)), or by binding of lysozyme itself. Representative fluorescence profiles are shown in Fig. 3 and the complete data are summarized in Table 1.

Analysis of double transgenic mice clearly demonstrated that the majority of B cells expressing the transgene-encoded receptor were not clonally deleted. At six weeks of age there was no reduction in the frequency or number of spleen cells expressing a-allotope heavy chains in the double-transgens, nor was there a decrease in the frequency or numbers of lysozyme-binding cells, compared with the Ig-transgenic littermates (Table 1). The amount of IgM expressed on the B-cells was, however, dramatically and selectively reduced (Fig. 3b, c). The modal fluorescence of double-transgenic B cells stained with a rat monoclonal antibody that binds equally well to a- and b-allototype IgM was an average of 3.8% that of the B-cells in the control Ig-transgenic littermates and 42% that of splenic B-cells in nontransgenic mice (Table 1). In contrast, the levels of IgD were unaltered. The selectivity of the effect for IgM was further demonstrated by the lack of any detectable change in the expression of other surface molecules such as B220, J11D, La, Ly-1 or Mac-1. At eight weeks of age these differences in immunoglobulin phenotype were still evident, and were observed in bone marrow and lymph nodes as well as in the spleen. A decline in the number and frequency of lysozyme-specific B cells in the spleens of double-transgenic versus Ig-transgenic littermates was, however, also apparent. This may be age-related and could reflect a lack of exogenous antigenic stimulation of lysozyme-specific B cells, an altered distribution amongst the lymphoid tissues, or terminal differentiation. But we cannot exclude the alternative possibility that a proportion of B cells may have been deleted (for example, those with high densities of surface IgM).

To exclude the possibility that the observed changes in secretion or surface expression of immunoglobulin in the double-transgenic mice might be due to production of significant amounts of lysozyme by the B cells, chimaeras were constructed by injecting MD-3 Ig-transgenic bone marrow cells into lethally irradiated ML-5 lysozyme-transgenic recipients or non-transgenic littermates. Analysis of the chimaeras three weeks later showed that the Ig-transgenic expressing B-cells in the lysozyme-transgenic recipients displayed the same phenotypic changes and absence of spontaneous immunoglobulin secretion as seen in the double-transgenic mice.

In view of the absence of anti-lysozyme antibody secretion and the persistence of lysozyme-binding B-cells in an altered phenotypic form, the next step was to determine whether the B cells in the double-transgenic mice had undergone a persistent functional change. A small number (10\(^6\)) of spleen cells from either a nontransgenic, Ig-transgenic, or double-transgenic mouse were injected into irradiated C57BL/6 recipients, together with an excess (5 x 10\(^6\)) of spleen cells from horse red blood cell-primed C57BL/6 mice as a source of helper T cells. Half of the recipients were injected with lysozyme covalently coupled to horse red blood cells at the time of transfer while the remainder served as controls. As shown in Fig. 5, moderate amounts of anti-lysozyme antibody were secreted in recipients of Ig-transgenic spleen cells, provided they were boosted with antigen (HEL-HRBC). In contrast, the titres in recipients of ‘double-transgenic’ spleen cells were 25-fold lower, and only marginally above the background levels in recipients of non-transgenic C57BL/6 spleen cells. The B cells from these double-transgenic mice therefore appear to respond poorly to stimulation with antigen in the presence of T cell help even though the
phenomenon seems to be dependent on multivalency. Given the flexibility of the model with respect to antigen presentation, and the feasibility of manipulating the T-cell repertoire in a similar fashion, it should be possible to resolve whether the apparent differences in our data compared to that of others reflect a true dichotomy in tolerance between T cells and B cells or simply different responses to 'weak' versus 'strong' self-antigens common to both subsets.

A second possible implication of the changes observed in the double-transgenic mice is that anti-lysozyme B cells expressing high levels of surface IgM are deleted, and this allows the development of a variant population with low levels of IgM but unchanged levels of IgD. Experiments to determine the molecular basis and reversibility of the decreased surface IgM expression should help to resolve this issue.

The failure of anti-lysozyme B cells from the double transgenic mice to respond efficiently to antigen and T-cell help (Fig. 5) suggests that they have undergone a persistent functional change. Further support for this conclusion comes from the striking alteration in surface immunoglobulin phenotype observed on FACS analysis (Table 1 and side infra). On the other hand, the findings do not allow us to distinguish between the various mechanisms which might bring about such a change. Thus a persistent functional change in the B-cell pool in theory could be induced either by encounter with lysozyme at an early stage of development (clonal anergy), or by interaction with antigen specific suppressor T-cells, or regulatory elements of an idiotypic network. Adaptive transfer of different subsets of cells at various stages of development together with the use of different lines of Ig-transgenic mice should help to distinguish between these alternatives.

The loss of surface IgM with persistent expression of IgD on the B cells of double-transgenic mice was a completely unexpected finding, and raises interesting questions as to the role of these immunoglobulin isotypes in tolerance induction. A negative correlation between surface IgD and susceptibility to tolerance has often been hypothesized because of the delayed appearance of IgD during B-cell maturation, and the sensitivity of immature or mature B-cells lacking IgD to inactivation by multivalent ligands in vitro10–17. On the other hand, the picture has been complicated by the absence of significant differences in the signalling properties of IgM and IgD for generation of intracellular second messengers, antigen uptake and processing, or B-cell activation in vitro18. The striking change in IgM/IgD receptor ratio induced in the double-transgenic mice (Fig. 3) provides further evidence for a differential role of IgD and IgM in B-cell tolerance, although the persistent expression of IgD on the tolerant B cells appears to contradict the hypothesis that IgD confers resistance to tolerance15. The precise role of the two isotypes, in particular whether the change in receptor status is a cause or an effect of the functional silencing in the double-transgenic B cells, will clearly be an important direction for future studies.

At first sight it is surprising that the selective downregulation of IgM demonstrated here has not been observed in studies of tolerance in vitro. Anti-IgM antibodies or antigen have been shown either to cause irreversible modulation of all surface immunoglobulin (clonal abortion) or to affect function but not surface immunoglobulin expression (clonal anergy)19–26. Selective downregulation of IgM on anergic B cells may have been overlooked, however, since IgD is not expected to be present on very immature B cells and has not been analysed. Intriguingly, the closest counterpart to the phenotypic changes in the B cells from the double-transgenic mice is found in normal B cell maturation. Indeed, the IgM<sup>+</sup>/IgD<sup>-</sup> phenotype of the B cells in the immunoglobulin-only transgenic littermates is similar to the phenotype of immature B cells which predominate in the spleens of one to two week-old mice, while the IgM<sup>+</sup>, IgD<sup>-</sup> phenotype of the double-transgenic B cells is similar to the mature B cells predominant in spleens of older mice21 (Fig. 3).
The experimental strategy of mating antigen-transgenic mice with antigen-receptor transgenic mice offers many advantages. Most importantly, it allows tolerance to be studied at the level of the whole animal, and yet provides a bridge between in vivo processes and the growing body of molecular and in vitro immunological data. Second, the induction of tolerance by a transgenic self antigen more accurately reproduces the physiological situation than does administration of exogenously synthesized antigens, particularly in view of the evidence that exogenous antigens may not be presented in association with class I major histocompatibility molecules. Third, experimental manipulation of the structure of the transgenic self antigen, tissue site of synthesis, timing of expression, or concentration can be achieved either by altering the microinjected construct or simply by crossing mice with different integration sites. Finally, the transgenic antigen receptor can be varied, for example by altering the fine specificity, isotype, or transmembrane domains encoded by the microinjected immunoglobulin genes.

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