Breakdown of Tolerance to a Neo-Self Antigen in Double Transgenic Mice in Which B Cells Present the Antigen

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Transgenic (Tg) mice expressing a foreign Ag, hen egg lysozyme (HEL), under control of the αA-crystallin promoter ("HEL-Tg" mice) develop immunotolerance to HEL attributed to the expression of HEL in their thymus. In this paper we analyzed the immune response in double (Dbl)-Tg mice generated by mating the HEL-Tg mice with Tg mice that express HEL Abs on their B cells ("Ig-Tg" mice). The B cell compartment of the Dbl-Tg mice was unaffected by the HEL presence and was essentially identical to that of the Ig-Tg mice. A partial breakdown of tolerance was seen in the T cell response to HEL of the Dbl-Tg mice, i.e., their lymphocyte proliferative response against HEL was remarkably higher than that of the HEL-Tg mice. T-lymphocytes of both Dbl-Tg and Ig-Tg mice responded to HEL at concentrations drastically lower than those found stimulatory to lymphocytes of the wild-type controls. Cell mixing experiments demonstrated that 1) the lymphocyte response against low concentrations of HEL is due to the exceedingly efficient Ag presenting capacity of the Ab expressing B cells and 2) breakdown of tolerance in Dbl-Tg mice can also be attributed to the APC capacity of B cells, that sensitize in vivo and stimulate in vitro populations of T cells with low affinity toward HEL, assumed to be escapees of thymic deletion. These results thus indicate that T cell tolerance can be partially overcome by the highly potent Ag presenting capacity of Ab expressing B cells. ***The Journal of Immunology*****, 2000, 164: 4594–4600.**

Analysis of the immune response in transgenic (Tg)[2] animals has yielded pivotal information concerning immunotolerance to self or non-self Ags (reviewed in Refs. 1–5). These studies revealed that transgenically expressed neo-self Ags may affect the immune system by three different mechanisms, clonal deletion, anergy, and ignorance (5). Both B- and T-lymphocytes are affected by these mechanisms, which take place either in the central immune organs (bone marrow and thymus, respectively) (6, 7), or in the periphery (8, 9). These studies have also shown that the neo-self Ag effect depends on three factors, the Ags concentration, its form, and its anatomical location (5).

A large volume of data has been produced by C. C. Goodnow and his collaborators concerning the fate of B cells specific to a neo-self Ag in Tg mice expressing this Ag (reviewed in Refs. 5 and 10). Their research employed double-Tg ("Dbl-Tg") mice generated by mating Tg mice in which hen egg lysozyme (HEL) was present ubiquitously (11–13) or in a single organ (14), with Tg mice in which the majority of B cells produced surface-bound and secreted Abs against this Ag ("Ig-Tg" mice). However, little information has been collected in these studies concerning the fate of T cells and the mode whereby Dbl-Tg mice respond to immunization with HEL.

In another study (15), we have examined the immune response of Tg mice that express HEL under transcriptional control of the lens αA-crystallin promoter. These mice, designated "HEL-Tg," were found to develop tolerance, apparently due to the expression of HEL in their thymus (15). The present study examined the immune profile of Dbl-Tg mice generated by mating our HEL-Tg mice with the Ig-Tg mice generated and used by Goodnow's group. Examination of the immune system of these Dbl-Tg mice showed that their B cell compartment was essentially unaffected, whereas the T cell compartment exhibited a partial breakdown of tolerance. Further analysis indicated that this breakdown of tolerance in the Dbl-Tg mice could be attributed to the highly potent Ag presenting capacity of B cells that sensitize and stimulate T-lymphocytes with low affinity toward HEL.

Materials and Methods

Mice

HEL-Tg mice were generated by placing the coding region of HEL plasmid KLK (a generous gift from C. C. Goodnow, Stanford University, Stanford, CA) under the transcriptional control of the murine αA-crystallin promoter. The transgene was excised from the plasmid and injected into FVB/N single cell embryos to create this mouse line as described in more detail elsewhere (15). Ig-Tg mice, in which the majority of B cells express IgM and IgD Abs/receptors for HEL (11), were kindly provided by C. C. Goodnow. These mice were maintained on a C57BL/6 (B6) background using wild-type (WT) mating mice purchased from The Jackson Laboratory (Bar Harbor, ME). Dbl-Tg mice were generated by mating HEL-Tg and Ig-Tg mice. All experiments recorded here were conducted with mice on the $(FVB/N \times B6)F_1$ background. The mice were housed under specific pathogen-free conditions at the facility of Biocon (Rockville, MD). All procedures with mice were conducted in compliance with the National Institutes of Health Resolution on the Use of Animals in Research. Similar to HEL-Tg mice on the FVB/N background (15), all HEL-Tg mice on the F_1 background had dystrophic eyes with disruption of the lens fibers and distortion of the lens capsule (see Fig. 7*B*).

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[2] Abbreviations used in this paper: Tg, transgenic; HEL, hen egg lysozyme; Dbl-Tg, double-transgenic; WT, wild type.

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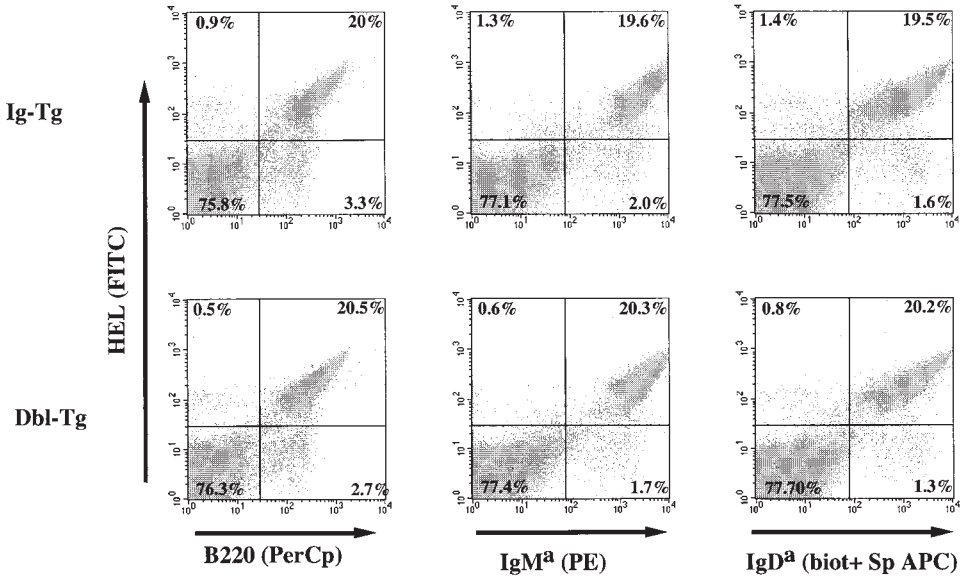

FIGURE 1. The B cell population of Dbl-Tg mice closely resembles that of the Ig-Tg mice. Spleen cells of the two mouse lines were stained for B cell-specific surface markers, B220, IgD and IgM, using specific Abs. In addition, the presence of HEL Ab on the surface was detected by incubation with labeled HEL, as detailed in *Materials and Methods*. A very similar staining pattern was obtained in another experiment.

FACScan analysis of splenocytes

Spleen cell suspensions were depleted of erythrocytes by treatment with ACK lysis buffer (BioWhittaker, Walkersville, MD) and were then stained as follows: 10^6 splenocytes were incubated with 0.2 μg of FITC-labeled HEL together with anti-CD45R/B220 PerCP-labeled Ab (clone RA3-6B2), anti-IgM^a PE-labeled Ab (clone DS-1), and anti IgD^a biotin-labeled Ab (clone AMS 9.1) (all Abs are from PharMingen, San Diego, CA). After a 20-min incubation at room temperature, cells were washed twice in PBS-1% BSA and cells were incubated for 15 min with APC-labeled streptavidin (Caltag, Burlingame, CA). Following additional washing, the cells were fixed in 1% paraformaldehyde and analyzed on a FACSCalibur Cytometer equipped with a 488 and 633 lasers (Beckman Dickinson, San Jose, CA). Acquisition and analysis were performed using CellQuest software (Becton Dickinson). Bivariate plots generated by the four-color staining are shown as HEL vs B220, IgM^a, and IgD^a. The quadrant statistics for 51,000 lymphocytes are indicated in the corresponding quadrant.

Immunization

Unless indicated otherwise, mice were injected with 25 μg HEL (Sigma, St. Louis, MO), emulsified in CFA containing *Mycobacterium tuberculosis* at 2.5 mg/ml (Difco, Detroit, MI). The emulsion, in a volume of 0.2 ml, was injected s.c. into the tail base and the two thighs.

Ab measurement

Serum Ab levels were measured 14 days postimmunization by ELISA, as described elsewhere (16). Microplate wells were coated with 300 ng HEL and bound Ab was detected by peroxidase-conjugated goat Abs against murine IgG1, IgG2a, or IgM (Southern Biotechnology Associates, Birmingham, AL). The data are presented as OD absorbance at 405 nm. All tests were conducted in duplicate, with individual values differing from the means by $\leq$10%.

Lymphocyte proliferation assays

Draining lymph nodes and spleen were collected 14 days following immunization, and their cells were tested for proliferative response against a series of HEL concentrations, as detailed elsewhere (17). In brief, 3×10^5 lymphoid cells were cultured, in triplicate or quadruplicate, with or without stimulants, in a final volume of 0.2 ml RPMI 1640 medium supplemented with a serum replacement, HL-1 (HYCOR, Irvine, CA), 2-ME (50 μM), and antibiotics. After incubation for 72 h, the cultures were pulsed with [^{3}H]thymidine (0.5 μCi/10 μl/well) for an additional 16 h and the incorporated radioactivity was measured by a scintillation counter. The data are expressed as mean Δ cpm values. Mean incorporation in unstimulated control cultures ranged between 392 and 3321 cpm. Variations among individual cultures were routinely $\leq$15% than the means.

Mixed cell cultures

Mixed cell cultures consisted of lymphoid cells from different mouse donors, at the indicated ratios and concentrations. Irradiated cells were exposed to 3000 rads. B cells were purified by the magnetic bead system of Miltenyi Biotech (Auburn, CA), following the manufacturer's instructions and using MACS CD19 Microbeads and an LS$^+$/VS$^+$ column. The purified cell suspension consisted of $\geq$95% B cells, as determined by FACS analysis. T cells were enriched by the mouse T cell Enrichment Columns (R&D Systems, Minneapolis, MN), following the manufacturer's instructions. The enriched fraction contained $\geq$85% CD3$^+$ cells by FACS analysis.

Cytokine assays

Spleen cells collected 14 days postimmunization were cultured in 24-well plates at 5×10^6/ml of the medium cited above, with or without stimulants. Supernatants were collected following incubation for 24 h (IL-2) or 48 h (IL-4 and IFN-γ) and stored at -70°C until use. Levels of all cytokines were measured by capture ELISA, using kits provided by Endogen (Woburn, MA).

Histological analysis

Eyes were collected from euthanized mice and histological sections were prepared and stained with hematoxylin and eosin using routine procedures.

Results

The B cell compartment is minimally affected in Dbl-Tg mice: surface markers analysis

Fig. 1 shows the FACScan analysis of splenocytes of the Dbl-Tg mice and their controls, the Ig-Tg mice. B-lymphocytes were stained with Abs against three surface markers, B220, IgD^a, and IgM^a, whereas their Ag specificity was depicted by their positive staining with HEL. As seen in this figure, the patterns of staining of cell suspensions from the two mouse lines were very similar, indicating that the presence of HEL in the Dbl-Tg mice had little effect if any on the B cell compartment of these animals.

Ab production in the Dbl-Tg mice resembles that in the Ig-Tg mice

Further evidence to indicate that the B cell compartment in the Dbl-Tg mice was not affected by the presence of HEL in these mice was obtained by comparing the Ab production in these animals to that in three other types of mice: Ig-Tg, HEL-Tg, or the WT littermate controls (Fig. 2). All mice were immunized with HEL 14 days before being assayed. The mouse sera were tested for HEL Abs of three isotypes: IgM, IgG2a, and IgG1. Both Ig-Tg and Dbl-Tg mice produced high levels of IgM HEL Abs before immunization (i.e., constitutive Ab production; see Ref. 11) (data not shown). Immunization of these mice with HEL had no detectable

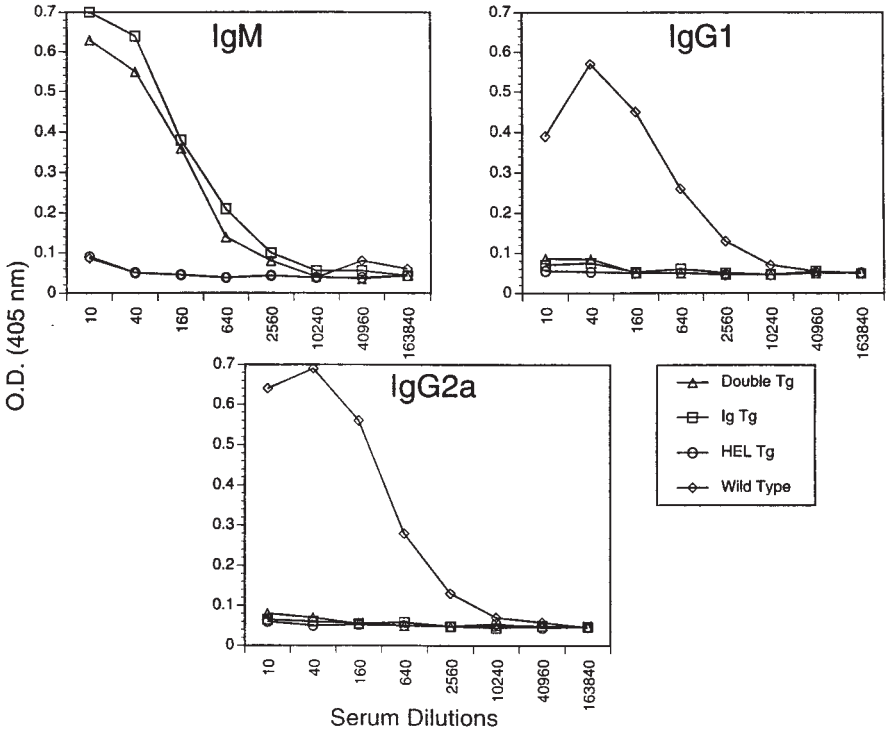

FIGURE 2. Dbl-Tg mice resemble their Ig-Tg controls in production of constitutive IgM Ab to HEL, with no isotype switch following immunization. Serum samples were collected 14 days postimmunization, and Ab levels of the different isotypes were measured by ELISA, using enzyme-conjugated goat Ab, as detailed in *Materials and Methods*. The curves represent titration results of representative individual mice from each group. Similar results were obtained in three other experiments, testing a total of at least eight mice of each line.

effect on the titer of these Abs, nor did it make the typical switch to the IgG isotype; only IgM Abs were found in sera of these two mouse lines (Fig. 2). In contrast, WT mice showed no IgM Ab, but produced high levels of both IgG2a and IgG1 Abs. Unlike the other mouse lines, HEL-Tg mice exhibited tolerance and did not produce any significant levels of HEL Abs, in line with our previous observation (15).

Partial breakdown of tolerance by the T cell compartment in Dbl-Tg mice

The cellular responsiveness toward HEL of the different mouse lines was determined by the proliferation response of their splenocytes following immunization with the Ag at 25 or 1 μg/mouse. Fig. 3 summarizes a representative experiment.

Fig. 3*A* shows the response of mice immunized with the 25 μg dose. WT mouse lymphocytes reacted well to the Ag, but only at relatively high concentrations, i.e., 1 μg/ml or higher. Ig-Tg cells, on the other hand, responded vigorously at a wide range of Ag concentrations, with high levels of proliferation being recorded in cultures stimulated with HEL at concentrations as low as 10^{-5} μg/ml. In line with our previous observation (15), lymphocytes from HEL-Tg mice exhibited tolerance against HEL, as depicted by the lack of response to the Ag at most concentrations; only the highest HEL concentration tested, 100 μg/ml, stimulated low magnitude proliferation. Of particular interest is the responsiveness of splenocytes from Dbl-Tg mice. Unlike the immunotolerance observed in their HEL-Tg littermates, cells from the Dbl-Tg animals responded well to HEL, albeit with levels lower than those of Ig-Tg cells. Importantly, Dbl-Tg lymphocytes also responded to HEL at a wide range of concentrations, with the lowest stimulatory concentration being $\sim 10^{-2}$ μg/ml, i.e., more than 100-fold lower than that of WT mouse cells.

The lymphocyte responses of mice from the four lines following immunization with 1 μg of HEL are shown in Fig. 3*B*. Only Ig-Tg and Dbl-Tg mice responded significantly when immunized with

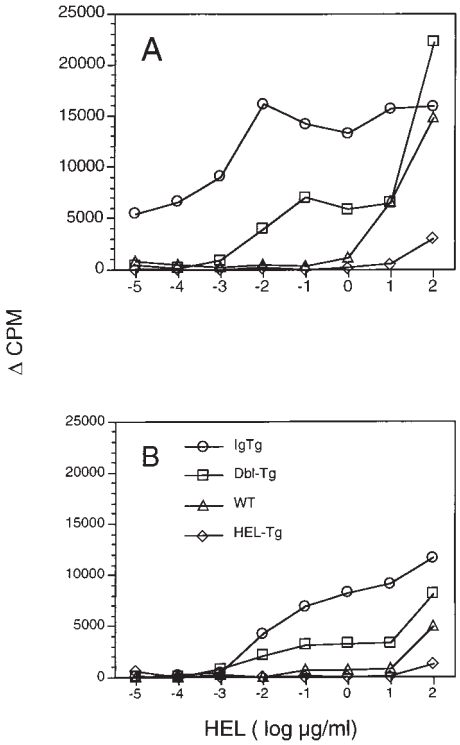

FIGURE 3. Partial breakdown of cellular immunotolerance to HEL in Dbl-Tg mice. Mice of the four lines were immunized with HEL at 25 μg (*A*) or 1 μg (*B*) per mouse and 14 days later their spleen cells were tested for proliferation against HEL at the indicated concentrations. The curves represent mean values of thymidine incorporation of representative individual mice of each group, stimulated with HEL at the indicated concentrations. A second experiment with these two immunizing doses yielded similar observations and the same pattern of responsiveness by cells of the four mouse lines was seen in other repeated experiments with mice immunized with 25 or 50 μg of HEL.

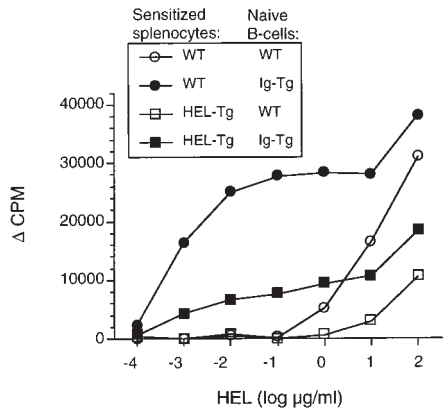

FIGURE 4. Naive Ig-Tg B cells modify the response to HEL of WT or HEL-Tg lymphocytes. Splenocytes from WT or HEL-Tg mice, collected 14 days following immunization with HEL, were stimulated in culture with different concentrations of HEL, as indicated, in the presence of purified B cells from naive WT or Ig-Tg mice. Cultures, in triplicate, consisted of 3×10^5 spleen cells and 1×10^5 purified B cells, as indicated. The curves represent mean thymidine incorporation in cultures of individual mice of a representative experiment, stimulated with HEL at the indicated concentrations. Very similar results were obtained in two other repeated experiments.

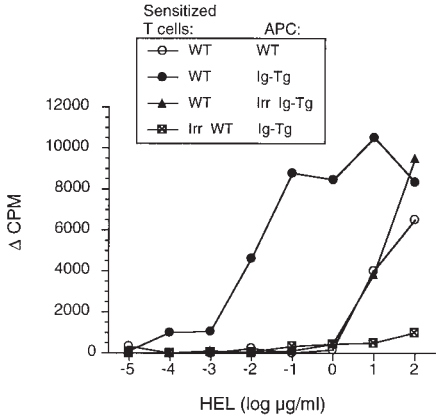

FIGURE 5. Elimination of B cell function by irradiation destroys the APC activity of naive Ig-Tg splenocytes. Spleen cells of mice immunized 14 days previously with HEL were cultured alone or with naive Ig-Tg splenocytes and stimulated by HEL at different concentrations, as indicated. Certain cell suspensions, as indicated ("Irr"), were irradiated (3000 rad) before being cultured. Cultures consisted of 3×10^5 spleen cells from immunized mice and 3×10^5 splenoytes from naive WT or Ig-Tg donors. The same pattern of responses was seen in a repeated experiment.

this low Ag dose, and their response magnitudes were substantially lower than those obtained following immunization with the 25 μg dose (Fig. 3*A*). In addition, cells from mice immunized with 1 μg responded to a range of HEL concentrations narrower than that stimulatory for lymphocytes of mice sensitized with the 25 μg dose.

The responsiveness to HEL of splenocytes from the Dbl-Tg mice indicates that the immune system of these animals overcame in part the tolerogenic mechanism(s) triggered by the transgenic expression of HEL in these mice. The following experiments were aimed at investigating this phenomenon.

APC activity of B cells transgenically expressing HEL Abs

When expressing specific Ab on their surface, B cells become exceedingly efficient APCs for the corresponding Ag (18, 19). Therefore, we hypothesized that the breakdown of tolerance in Dbl-Tg mice is due to the APC activity of B cells of these animals. This notion was examined by testing B cells from naive Ig-Tg mice for their capacity to alter the pattern of response of lymphocytes of other mouse lines. Fig. 4 summarizes a typical experiment in which B cells from naive Ig-Tg spleens were added to cultures of splenocytes from HEL-immunized WT or HEL-Tg mice. B cells from naive WT mice were used as controls in these experiments. Adding naive WT B cells had no effect on the pattern of response to HEL of spleen cells from the immunized mice: similar to responses recorded above (Fig. 3), sensitized WT splenocytes responded well to HEL, but only at concentrations of ≥ 1 μg/ml, whereas immunized HEL-Tg spleen cells responded poorly and only at 10 or 100 μg/ml. In contrast to WT B cells, naive Ig-Tg B cells had profound effects on the response of the immunized splenocytes, mainly by widening their range of response to HEL and shifting the dose response curves to concentrations lower by 4–5 orders of magnitude. The effect of naive Ig-Tg B cells was particularly dramatic on WT spleen cultures, but it is noteworthy that even the low response of HEL-Tg lymphocytes was broadened by ~4 orders of magnitude.

The identification of B cells as the APC responsible for enabling the response to low HEL concentrations was further confirmed by testing the activity of naive Ig-Tg whole spleen cell suspension following irradiation at 3000 rad. This irradiation dose selectively destroys the APC capacity of B cells (20). As shown in Fig. 5, naive Ig-Tg whole spleen cell suspension resembled the purified B

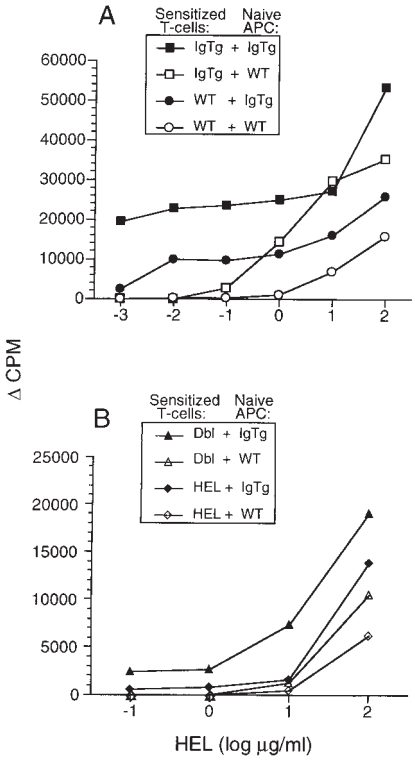

FIGURE 6. Pattern of T cell response against HEL is determined by the APC. Enriched T cell populations were prepared from lymph nodes of WT, Ig-Tg, HEL-Tg, or Dbl-Tg mice 14 days following immunization with HEL. Cultures, in triplicate, consisted of 2×10^5 enriched T cells and 2×10^5 spleen cells from naive WT or Ig-Tg mice, serving as APC. The curves show mean values of thymidine incorporation of individual mice of a representative experiment, stimulated with HEL at the indicated concentrations. Two other repeated experiments yielded very similar results.

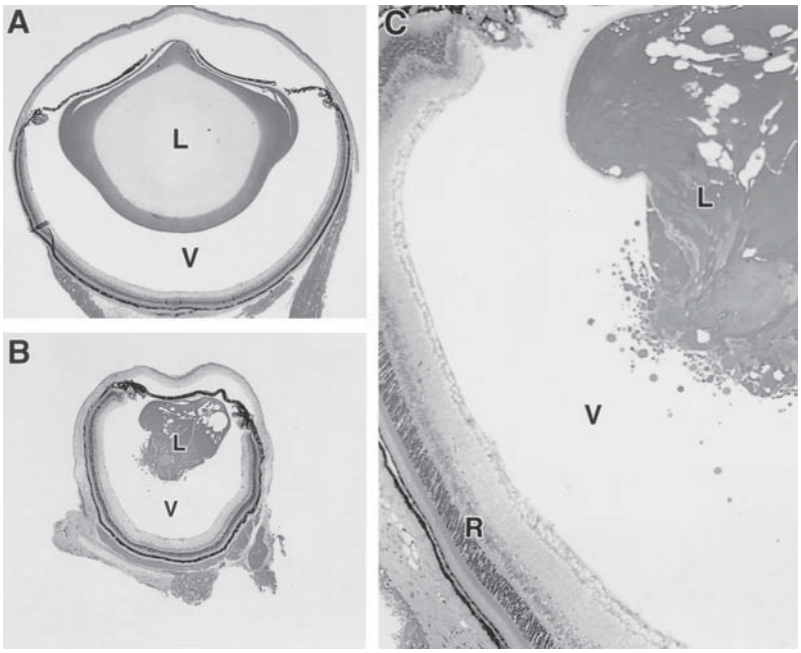

FIGURE 7. No inflammation is seen in eyes of Dbl-Tg mice immunized with HEL. *A*, Section of an eye from a WT control mouse, demonstrating the typical morphology of a normal eye (×25). An identical normal morphology is seen routinely in sections of Ig-Tg mice (not shown). *B*, Section at a low magnification (×25) of an eye of a Dbl-Tg mouse immunized with HEL. The eye is dystrophic and remarkably smaller than the WT control eye shown in *A*. The characteristic morphology of the lens is completely disrupted and leakage of lenticular material is seen at the posterior pole. Importantly, no inflammation is observed in any area of this eye. *C*, A portion of the eye shown in *B*, at a higher magnification (×200). Shown here are the disrupted lens and its leaking material, as well as the inflammation-free vitreous. L, lens; V, vitreous; R, retina (hematoxylin-eosin).

cell fraction in widening the response of HEL-sensitized WT lymphocytes toward much lower HEL concentration. This effect of Ig-Tg spleen cells was completely eliminated, however, by irradiation, thus confirming our assumption that the potent APC activity of Ig-Tg splenocytes is totally provided by the B cell population.

Breakdown of tolerance: analysis at the T cell level

To further learn about the mode of action whereby B cells abrogate tolerance in Dbl-Tg mice, we examined the effect of APC from naive Ig-Tg or WT mice on the proliferative response of T cells from Dbl-Tg mice, their two single-Tg controls, and WT littermates. The data of a representative experiment are shown in Fig. 6. Unlike the striking difference between the pattern of response of whole spleen cell cultures of WT and Ig-Tg (Fig. 3), the T cell fractions of these mice responded similarly when cultured with naive APC (Fig. 6*A*). When cultured in the presence of WT APC, both T cell populations proliferated only when stimulated with high concentrations of HEL (10 and 1 μg/ml, respectively). However, a profound shift toward lower HEL concentrations was seen when these cells were cultured with Ig-Tg APC. A similar pattern of response, but with lower levels of proliferation, was seen with T cells from Dbl-Tg and HEL-Tg mice when cultured with the two types of APC (Fig. 6*B*). It is noteworthy that Dbl-Tg T cells responded with remarkably higher levels than the HEL-Tg T cells when cultured with either Ig-Tg APC (at all concentrations), or WT APC (mainly at the highest HEL concentration). This observation suggests that the partial breakdown of tolerance seen with whole spleen cells of Dbl-Tg mice can be attributed to the APC of these mice promoting both the in vivo and in vitro responses.

No inflammation is detected in eyes of Dbl-Tg mice

Despite the presence of high concentrations of HEL Abs (Fig. 2) and moderate levels of cellular immunity to HEL (Fig. 3*A*) in the immunized Dbl-Tg mice, no inflammatory changes could be detected in eyes of these mice (Fig. 7). It is noteworthy that due to the excessive expression of HEL in the lens, eyes of the Dbl-Tg mice were dystrophic, with disruption of the lens capsule and distortion of the lens fibers (Fig. 7). It was previously demonstrated (21) that these morphological changes allowed HEL release from the lens.

Cytokine production by Dbl-Tg and other mouse lines

The absence of inflammation in eyes of Dbl-Tg mice that developed cellular response to HEL could be explained by the incapacity of the responding cells to produce proinflammatory Th1 cytokines; Ag presentation by B cells was reported to skew the immune response toward the Th2 type (22, 23). To test this hypothesis, spleen cells from Dbl-Tg, Ig-Tg, and WT control mice, immunized with HEL, were examined for cytokine production in culture (Fig.

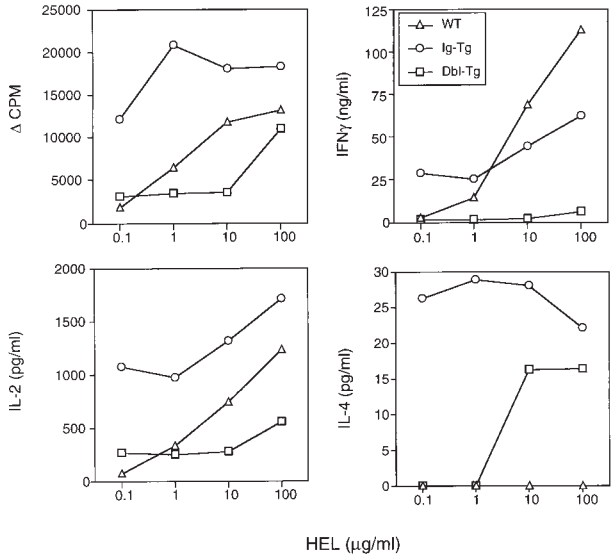

FIGURE 8. Different patterns of cytokine production by lymphocytes of Dbl-Tg, Ig-Tg, and WT mice. Spleen cells of the three mouse lines were collected 14 days following immunization with HEL and tested for proliferation and production of IL-2, IL-4, and IFN-γ when cultured with HEL at different concentrations. Lymphocytes of two or three mice of each line were pooled for these tests. No cytokines were detected in cultures of any of the mouse lines when incubated without stimulants (not shown). The same pattern of selective cytokine production was seen in two other experiments, one of which was conducted with draining lymph node cells.

8). Production of IL-2 by lymphocytes from the three mouse lines paralleled their pattern of proliferative responses. Differences were found, however, between the proliferative responses and production of IFN-γ and IL-4 of the three mouse lines. WT cells produced high levels of IFN-γ, but failed to release any detectable IL-4. Dbl-Tg, on the other hand, produced negligible levels of IFN-γ but, interestingly, released moderate amounts of IL-4 but only when stimulated with high concentrations of HEL, i.e., 10 and 100 μg/ml. Ig-Tg lymphocytes produced moderate levels of IFN-γ, but high levels of IL-4, at all tested HEL concentrations (0.1–100 μg/ml).

Discussion

Data recorded in this paper demonstrate the unique features of the immunological profile of Dbl-Tg mice in which HEL is expressed under the αA-crystallin promoter and IgD and IgM HEL Abs are produced by the majority of B cells. The B cell compartment of the Dbl-Tg mice was not affected by the presence of HEL in these mice: both the pattern of B cell staining (Fig. 1) and the Ab production (Fig. 2) of the Dbl-Tg mice closely resembled those of the Ig-Tg mice. These observations differ from those made by C. C. Goodnow and his collaborators with Dbl-Tg mice generated by mating the Ig-Tg mice with Tg mice expressing high levels of soluble HEL in the liver (5, 10), or membrane-bound HEL on MHC class I positive cells in multiple organs (5, 12). Anergy of B cells and altered Ig expression was observed by these investigators in the former Dbl-Tg mice (5, 10), whereas deletion of the HEL-specific B cells was seen in the latter type of Dbl-Tg mice (5, 12). The difference between our findings and those of Goodnow's group can be attributed to the low levels of HEL in the Dbl-Tg mice in the present study. Indeed, no tolerance toward HEL by B cells was found by Goodnow and his coworkers in Dbl-Tg mice with HEL levels lower than 1.5 ng/ml in their blood (5, 13), or in Dbl-Tg mice in which HEL was selectively expressed on thyroid cell membranes (14). Likewise, no HEL could be detected in the serum of our HEL-Tg mice when using a method with a threshold of 1 ng/ml (15). It is noteworthy, however, that the HEL-Tg mice used to generate the DBL-Tg mice in our study resembled the Tg mice with high levels of soluble HEL in the study of Goodnow's group (13) in showing both cellular (Fig. 3*A*) and humoral (Fig. 2) unresponsiveness to HEL. We attribute the very effective tolerogenic mechanism in our HEL-Tg mice to the expression of HEL in the thymus of these mice and the putative ensuing clonal deletion of HEL-specific T cells (15). The finding that B cells are not affected in our Dbl-Tg mice (Figs. 1 and 2) suggests that the B cell tolerance in the HEL-Tg mice is probably due to deficiency in helper T cells. This notion is also in line with the observation that WT mice and rabbits immunized with self lens crystallins produce Ab, but exhibit T cell unresponsiveness against these autoantigens (24, 25). Thymic expression of crystallins (26, 27) is probably responsible for the T cell tolerance, whereas the large doses of self crystallins used in the cited studies (24, 25) are assumed to stimulate a small number of T cells that escape deletion and provide help to an unaffected population of B cells (see below).

A major finding in the present study is the partial abrogation of tolerance in the Dbl-Tg mice; unlike the tolerance in the HEL-Tg mice, the Dbl-Tg mice did develop substantial levels of cellular immune response against HEL (Fig. 3). Moreover, tolerance breakdown was observed even in Dbl-Tg mice following immunization with 1 μg HEL, a dose that produced only a slight immune response in WT controls. The breakdown of tolerance to HEL in the Dbl-Tg mice can be attributed to the exceedingly efficient APC capacity of B cells in these animals. This capacity was manifested here by both 1) the vigorous proliferative response of Ig-Tg splenocytes at strikingly low HEL concentrations (e.g., Fig. 3*A*) and 2) by the finding that naive Ig-Tg splenocytes or B cells profoundly enhanced the response of HEL-sensitized WT lymphocytes and enabled these T cells to resemble HEL-sensitized Ig-Tg cells in their response against low concentrations of the Ag (Figs. 4–6). Furthermore, T cells from Ig-Tg mice responded only to high concentrations of HEL when cultured with WT APC (Fig. 6). The highly potent Ag presenting capability of Ig-Tg B cells was recorded and studied by Kanost and McCluskey (18), who suggested that the Abs on these cells enhance the capture of HEL and modify its processing, thereby enabling the activation of T cells with low affinity toward this Ag. Therefore, it is proposed that by their highly potent APC activity the B cells in Dbl-Tg mice make it possible for the T cell compartment to partially overcome the tolerogenic mechanism in these animals. Tolerance is assumed to develop in HEL-Tg mice mostly by clonal deletion in the thymus (1, 2, 4, 6), a process that allows the escape of T cells with low affinity toward HEL (6, 28, 29). These low affinity T cells are assumed to get sensitized in vivo and become activated in vitro in the Dbl-Tg mice in which the Tg B-lymphocytes function as APC. When cultured with WT APC, however, these T cells of Dbl-Tg mice responded with lower proliferation levels and only against the high concentrations of HEL (Fig. 6*B*). A small number of lymphocytes also became sensitized against HEL in HEL-Tg mice. These T cells, which are also assumed to be escapees from the thymic deletion, responded only against the high concentrations of HEL (100 μg/ml) (Fig. 3*A*), but their response was enhanced and extended to lower Ag concentrations when Ig-Tg APC were added (Figs. 4 and 6*B*). The notion concerning the involvement of T cells with low affinity in the breakdown of tolerance in the Dbl-Tg mice is also in accord with data of other studies, showing that tolerance in Tg mice can be overcome by immunization with very high doses of the neo-self Ag (30), or by infusion of activated dendritic cells (31).

In addition to cell-bound Abs, the Ag presentation in Dbl-Tg and Ig-Tg mice could be promoted by circulating Abs in these mice. Mice of both lines produce constitutively high levels of HEL Abs, capable of forming immune complexes with the Ag and thus enhancing its capture by dendritic cells or macrophages (32–34).

Despite the presence of high levels of IgM Abs and lymphocytes sensitized against HEL, no inflammation was detected in HEL expressing eyes of the Dbl-Tg mice (Fig. 7). A similar observation was made by Goodnow's group in thyroids of Dbl-Tg mice generated by mating Ig-Tg mice with Tg mice expressing HEL on their thyroid cell membranes (14). These authors attributed the lack of thyroid inflammation to the physical barrier between blood and thyroid parenchyma (14). The eye, too, is partially sequestered from the immune system and, in addition, is equipped with multiple anti-inflammatory mechanisms (35–37) that could block or hamper the development of inflammation. Yet the nonpathogenicity of HEL-specific T cells in the Dbl-Tg mice could be attributed to a large extent to their incapacity to produce pro-inflammatory cytokines such as IFN-γ (Fig. 8). It is also noteworthy that lymphocytes from Dbl-Tg mice produced IL-4, but only when stimulated by high HEL concentrations (10 and 100 μg/ml). On the other hand, these lymphocytes proliferated and released IL-2 at much lower HEL concentrations (Fig. 8). These findings are in accord with the recent observation by Rogers and Croft (38), that IL-2 is the only cytokine produced by lymphocytes with low affinity to the Ag when stimulated with low Ag levels.

To conclude, our data show that when expressing specific Ab, B cells become highly potent APC, capable of overcoming tolerogenic processes of the T cell compartment. It is of note that this

activity of B cells may have a major adverse effect, by abrogating tolerance to self Ags and consequently, bringing about pathogenic autoimmunity (39).

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