Immunotolerance against a Foreign Antigen Transgenically Expressed in the Lens

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Purpose. To extend our knowledge concerning immunotolerance against autologous lens crystallins, transgenic (Tg) mice that express a foreign antigen in their lens were generated, and the immune response against the antigen in these mice was analyzed.

Methods. Conventional techniques were used to generate lines of Tg mice that express soluble (S-) or membrane-bound (M-) hen egg lysozyme (HEL) under the control of the αA-crystallin promoter. The presence of HEL in various organs was determined by the particle concentration fluorescence immunoassay (PCFIA), and reverse transcription-polymerase chain reaction technique was used to detect mRNA transcripts of the molecule. To examine the development of immunity (or tolerance), Tg mice and their wild-type controls were immunized with HEL (25 μg) in Freund's complete adjuvant and 14 days later were tested for immune response against the antigen. Cellular immunity was measured by the lymphocyte proliferation assay and cytokine production, and humoral immunity was determined by enzyme-linked immunosorbent assay.

Results. Eyes of the high copy number M-HEL Tg mice were dystrophic, with disrupted lens, whereas no morphologic changes were detected in the eyes of the other Tg mouse lines. All Tg mice exhibited tolerance to HEL by their cellular and humoral immune compartments. The state of immunotolerance to HEL was retained in the Tg mice for as long as 10 months after removal of the main depot of this protein, by enucleation. Measurable amounts of HEL were found in the eyes of all Tg mice, but the protein could not be detected in the serum or in other organs by the sensitive PCFIA (with a threshold of 1 ng/ml). Yet, HEL mRNA was found in the thymus of the Tg mice, suggesting that minute amounts of the protein are expressed in this organ.

Conclusions. The unresponsiveness to HEL in the Tg mice seems to be due to a "central" mechanism of tolerance, mediated by a minuscule amount of HEL in the thymus. Conversely, the much larger amounts of HEL in the peripheral depot, the eyes, play a minor role if any in the tolerogenic process. It is further proposed that a similar mechanism of central tolerance is responsible for the immunotolerance against autologous lens crystallins. (*Invest Ophthalmol Vis Sci.* 1998;39:2049-2057)

Lens crystallins are sequestered from the immune system by the lack of vasculature and a thick capsule, and were expected, therefore, to be highly autoimmunogenic. However, previously reported data[1,2] have shown that crystallins are poorly immunogenic when injected into animals of the same species ("allogeneic"). Thus, mice[1] and rabbits[2] immunized with allogeneic crystallins did not develop any T-cell response, and only low levels of antibodies were produced. Conversely, immunization of these animals with crystallins from other species ("xenogeneic") stimulated vigorous responses of the T- and B-cell compartments.[1,2] The lack of T-cell response to the allogeneic crystallins was attributed to "low zone tolerance" that was induced by the low level release of lens crystallins.[1]

The availability of transgene technology has provided new approaches to the analysis of the development of tolerance to self-antigens. In these studies foreign antigens are transgenically expressed under the promoter of autologous proteins, and the immune responses to the neo-self-antigens are measured. Immunotolerance against the foreign antigens was found in transgenic (Tg) mice that express these antigens under the promoter of ubiquitous proteins, such as albumin[3] or metallothionein,[4,5] and in Tg mice in which the foreign antigens were expressed locally, in organs such as the pancreas,[6-8] with limited access to the immune system. Development of tolerance in mice with local expression of the foreign antigens was attributed in early reports to mechanisms of "peripheral tolerance,"[6,7,9] but more recent data suggest that "central tolerance" plays a major role in the process.[8,10] In central tolerance, lymphocytes are deleted or anergized when exposed to specific antigens in the thymus, whereas in peripheral tolerance the process takes place in other organs.[9,11]

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The present study examined the immune responsiveness in Tg mice in which a foreign antigen, hen egg lysozyme (HEL), is expressed under the control of the αA-crystallin promoter. These Tg mice developed immunotolerance to HEL, and the finding of HEL mRNA transcript in their thymuses suggests that the state of tolerance can be attributed mainly to the presence of minute amounts of HEL in their thymuses. Moreover, the Tg mice expressing HEL primarily in their lens made it possible to show that removal of the peripheral depot of the antigen, by enucleation, did not abrogate the unresponsiveness to this protein.

Materials and Methods

Generation of HEL Transgenic Mice

The coding regions of HEL plasmids pMTH and KLK (kindly provided by C. C. Goodnow, Stanford University, Stanford, CA) were placed under the transcriptional control of the murine αA-crystallin promoter to generate constructs PRL1 and PRL2, respectively. The transgenes were excised from the plasmids and injected into FVB/N single-cell embryos to create Tg mice that express a soluble (S-HEL) form or a membrane-bound form of HEL (M-HEL) (PRL1, 1 line, and PRL2, 4 lines, respectively). Potential founders were screened for the presence of the transgene by Southern blot analysis. Positive offspring of the Tg founders were identified by a standard polymerase chain reaction (PCR) assay using HEL primers. Phenotypic changes in eyes of the Tg mice were examined using conventional histologic methods. All procedures with mice were carried out in compliance with the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research.

HEL and Total Protein Content in Tissues

Various organs were collected from HEL-Tg mice and their wild-type (WT) control littermates, at 6 to 8 weeks of age. The tissues were homogenized in ice-cold phosphate-buffered saline, and the homogenates were centrifuged at 500*g* for 30 minutes. Protein concentration in the supernatants was determined by the Coomassie Plus Protein Assay Reagent kit (Pierce, Rockford, IL). HEL concentration in the supernatants was determined by the particle concentration fluorescence immunoassay (PCFIA), as described in detail elsewhere.[12] Briefly, 0.9-μm polyester particles (Idexx Corp., Portland, ME) were coated with HEL-specific monoclonal antibody HyHEL10. Samples were incubated with the coated beads, and HEL binding was detected by incubating the beads with two different fluorescein isothiocyanate- conjugated HEL-specific antibodies, HyHEL5 and HyHEL9. The fluorescence intensity was read by a "Screen Machine" (Idexx Corp.).

Detection of HEL mRNA in Mouse Organs with Reverse Transcription–PCR

Total RNA from the tested organs was extracted using TRIzol reagent according to the procedure recommended by the manufacturer (GIBCO/BRL, Gaithersburg, MD). The cDNA were generated from 2 μg RNA in a 50-μl reaction using 500 U of SuperScriptII reverse transcriptase and oligo(dT)12-18 primer (GIBCO/BRL). The incubation was carried out at 44°C for 60 minutes. For purification, the resultant cDNA was subjected to LiCl precipitation and resuspended in 50 μl water. The PCR amplifications were performed in a Perkin-Elmer GeneAmp 9600 using 1 μl cDNA in a total volume of 50 μl with AmpliTaq Gold DNA polymerase, as recommended by the manufacturer (Perkin-Elmer Cetus, Norwalk, CT). The AmpliTaq Gold polymerase was activated at 95°C for 10 minutes, the PCR was performed for a total of 35 cycles, which consisted of denaturation at 94°C for 30 seconds, annealing at 63°C for 30 seconds, and extension at 72°C for 30 seconds, followed by a 10-minute extension at 72°C.

The primers used for reverse transcription (RT)-PCR analysis of HEL sequence were as follows: 5′-TTGGTGCTTTGCTTCCTGCCCCTG-3′ and 5′-TGCCGTTTCCATCGCTGACTGACGATC-3′. These primers are located in exons 1 and 3 of the HEL gene, and give an amplified fragment of 449 bp from mature HEL mRNA. As controls, primers 5′-GTGGGCCGCTCTAGGCACCAA-3′, and 5′-TCGTTGCCAATAGTGATGACTTGGC-3′, which correspond to a 655-bp fragment of mouse β-actin, were used to amplify cDNA samples. The amplified PCR fragments (35 μl) were electrophoresed on a 1.5% agarose gel containing ethidium bromide.

Immunization

All experimental mice were immunized with 25 μg HEL (Sigma Chemical Co., St. Louis, MO) emulsified in Freund's complete adjuvant containing *Mycobacterium tuberculosis* at 2.5 mg/ml (DIFCO, Detroit, MI). The emulsion, in a volume of 0.2 ml, was injected subcutaneously into the tail base and the two thighs.

Immune Responses

Immunized mice were tested 14 days after injection. Cellular immunity was assessed by the lymphocyte proliferation assay as detailed elsewhere.[13] In brief, 3×10^5 lymph node or spleen cells were cultured in triplicate, with or without stimulants, in a final volume of 0.2 ml RPMI-1640 medium supplemented with HL-1 (HYCOR, Irvine, CA), 2-mercaptoethanol (50 μM), and antibiotics. In certain cultures, as indicated, recombinant interleukin (IL)-2 (Proleukin, Cetus, Bridgeton, MD) was added to the culture medium, at a final concentration of 100 U per ml. After incubation for 72 hours, the cultures were pulsed with [^{3}H]thymidine (0.5 μCi/10 μl/well) for 16 additional hours, and the incorporated radioactivity was measured by a scintillation counter. The data are expressed as changes in counts per minute (Δcpm) or as stimulation index values (expressed as mean cpm in cultures with stimulant/mean cpm in unstimulated control cultures). Mean incorporation in unstimulated control cultures of lymph node cells ranged between 584 and 1601 cpm and that of splenocytes between 956 and 4831 cpm.

Cytokine production was measured in cultures of splenocytes from Tg or WT mice 14 days after immunization with HEL. Cultures were set up in 24-well plates and consisted of 5×10^6 spleen cells in 1 ml of the above medium, with or without stimulants, as indicated. Supernatants were collected after incubation for 24, 48, or 72 hours, and levels of cytokines were determined using commercial kits provided by Genzyme (Cambridge, MA; interferon [IFN]-γ), Promega (Madison, WI; transforming growth factor [TGF]-β_1), and Endogen (Woburn, MA; IL-2, IL-4, and IL-10).

Antibody levels were measured by enzyme-linked immunosorbent assay (ELISA).[14] Wells were coated with 300 ng HEL, and bound antibody was detected by peroxidase-conjugated goat anti-mouse IgG (ICN, Aurora, OH). The data are presented as optical density absorbance at 405nm.

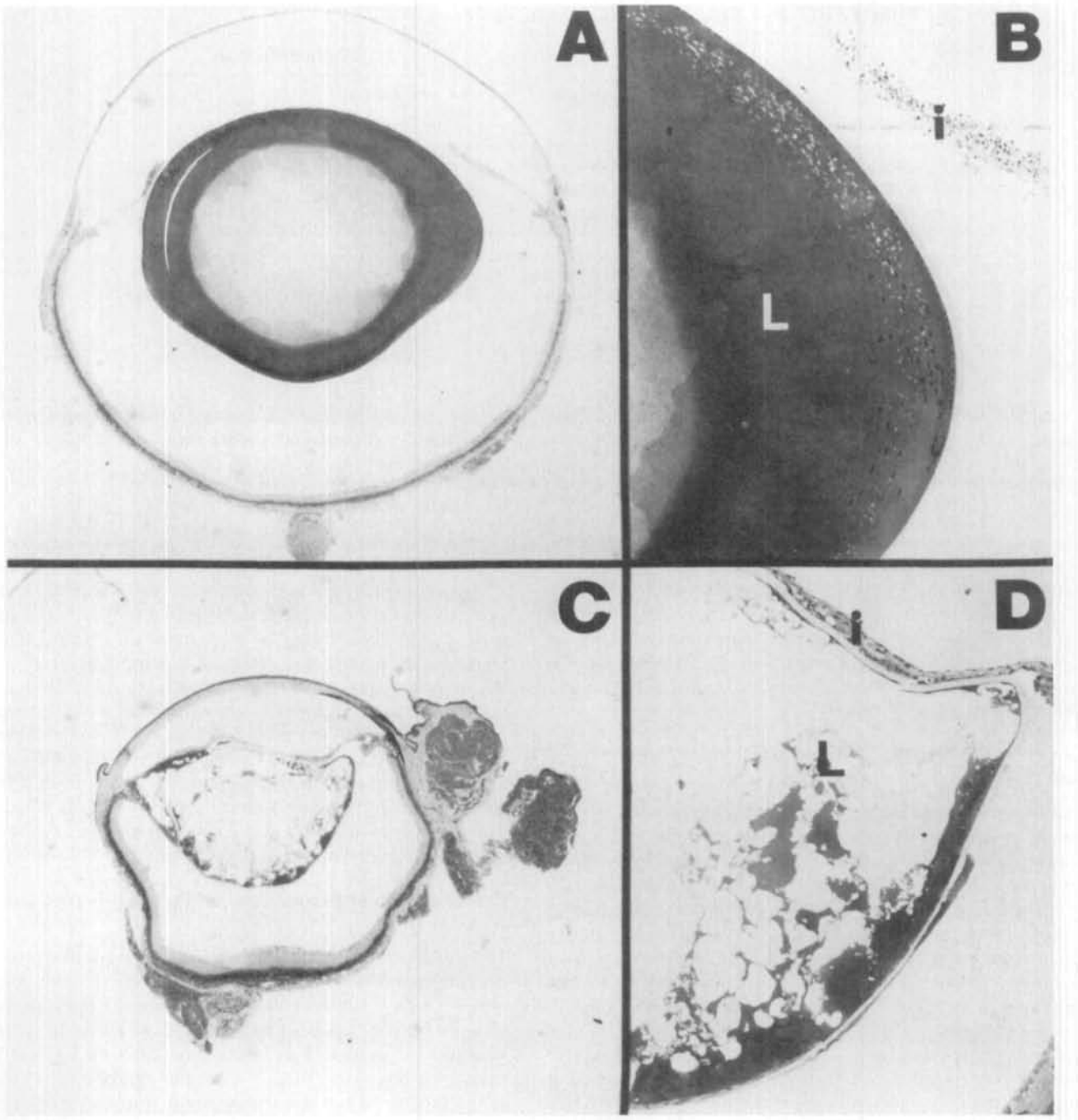

FIGURE 1. Morphologic changes in the eyes of membrane-bound form of hen egg lysozyme (M-HEL) high copy number transgenic (Tg) mice. Sections of an affected eye (**C**, **D**) are compared with those of a wild-type (WT) control (**A**, **B**). The eye of the Tg mouse is dystrophic and remarkably smaller than the eye of the WT control animal. The characteristic morphology of the lens is completely disrupted. However, no inflammation is detected in the affected eye. Hematoxylin-eosin; magnification, (**A**, **C**) ×25 and (**B**, **D**) ×100. L, lens; i, iris.

Enucleation of Mice

Transgenic mice and their WT littermate controls were bilaterally enucleated at approximately 4 weeks of age. The procedure was carried out on mice anesthetized with avertin (16 μl 2.5% solution/g body wt). Subgroups of mice were immunized at different times after enucleation, as indicated, and their immune responses were determined as described above.

RESULTS

Generation and DNA Analysis of Tg Founder Lines

Four lines of Tg mice expressing M-HEL and one line with S-HEL were generated. As determined by Southern blot analysis, two of the M-HEL lines had high copy numbers of the transgene, and the other two M-HEL lines had low copy num-

TABLE 1. HEL and Total Protein in Eyes of Tg Mice*

Tg Mouse Line	Total Protein/ Eye (μg)	HEL Concentration ng/eye	HEL Concentration ng/μg Total Protein
M-HEL (high†)	216.0 ± 39.9	90.0 ± 4.8	0.437 ± 0.170
M-HEL (low†)	1206.8 ± 187.5	13.0 ± 1.0	0.011 ± 0.002
S-HEL	1326.9 ± 122.2	107.9 ± 16.2	0.081 ± 0.009

M-HEL, membrane-bound form of hen egg lysozyme; S-HEL, soluble form of HEL; Tg, transgenic.

* All values are the mean ± SE of 2 to 6 individual mice of the corresponding mouse line. Extracts of whole eyes were prepared, and protein and HEL concentration were determined as detailed in the Materials and Methods Section.

† Transgene copy number.

bers. The S-HEL line showed a low copy number of the transgene.

Phenotypes of the Tg Mouse Lines

Eyes of the S-HEL line and the two low copy number M-HEL lines showed no detectable abnormality, either macroscopically or histologically. In contrast, all eyes of the high copy number M-HEL mice were dystrophic, and histologic examination revealed severe disruption of the lens fibers and distortion of the lens capsule (Fig. 1). It is noteworthy, however, that no inflammatory changes were detected in any of the dystrophic eyes, even in those of mice immunized with HEL in Freund's complete adjuvant (see below).

Expression of HEL in Eyes and Other Organs of Tg Mice

Samples of serum and extracts of various tissues of the Tg mice were tested for HEL using the highly sensitive PCFIA; the lowest detectable concentration of HEL measured by this assay, when added to normal mouse serum, was 1 ng/ml. No HEL could be detected by the PCFIA in the thymus, spleen, liver, heart muscle, or serum of the Tg mice (data not shown). In contrast, measurable amounts of HEL were found in the eyes of all tested Tg mice (Table 1). The data are presented as nanograms of HEL per eye and nanograms of HEL per microgram of total protein to indicate the substantial decline in size of eyes of the high copy number M-HEL. These dystrophic eyes had the higher concentration of HEL per microgram of protein, but their total HEL content resembled that of eyes of the S-HEL mice. The lowest concentrations of HEL were measured in eyes of the low copy number M-HEL mice.

Immunotolerance in HEL-Tg Mice: Cellular Response

Groups of Tg mice of the three types and their littermate WT controls were immunized with HEL in Freund's complete adjuvant and tested for cellular response against the antigen using the lymphocyte proliferation assay. Figure 2 summarizes data of several individual mice of the three Tg types and their WT controls; the same patterns of response were obtained with lymphocytes of at least five additional mice of each type. Although lymphocytes from WT mice responded vigorously to HEL, lymph node and spleen cells from the three types of Tg mice failed to show significant responses to this antigen. In contrast to their unresponsiveness to HEL, lymphocytes of all Tg mice resembled their WT controls in their response against tuberculin or concanavalin A (data not shown).

Immunotolerance in Tg Mice: Cytokine Production

Unresponsiveness to HEL in the Tg mice was also demonstrated by the diminished cytokine production by their splenocytes when stimulated in culture with HEL (Table 2). Transgenic spleen cells were particularly inferior to WT control cells in their production of the type 1 cytokines, i.e., IL-2 and IFN-γ. Only low levels of type 2 cytokines IL-4 and IL-10 were produced in WT control cultures in response to HEL, and the Tg cultures yielded even lower levels of these cytokines that were below the detection threshold of the assays. Stimulation with HEL had no effect on TGF-β_1 production by either WT or Tg cells. In contrast to their poor cytokine production when stimulated with HEL, Tg spleen cells resembled their WT controls in their production of cytokines when stimulated with the polyclonal mitogen concanavalin A (Table 2).

Cellular Unresponsiveness of Tg Mice Is Not Due to Anergy

To examine whether anergy plays a role in the unresponsiveness of lymphocytes to HEL observed in Tg mice, the response of these cells to HEL was tested in the presence of exogenous IL-2; this cytokine has been shown to reverse anergy in various systems.[15-17] As shown in Figure 3, cultures of WT and Tg mice both responded to IL-2 when the cytokine was added alone. Exogenous IL-2 also enhanced the response of WT cells against all tested HEL concentrations, with the response curve paralleling the curve obtained without IL-2. A parallel was also seen between the response curves of Tg cells cultured with different concentrations of HEL with or without IL-2. Exogenous IL-2 had no additional effect on Tg cultures at the low HEL concentrations (0.1 μg/ml and 1.0 μg/ml), and moderately increased the response of cultures with HEL at 10 μg/ml and 100 μg/ml. These data suggest that anergy plays no significant role in inducing the state of unresponsiveness to HEL in the Tg mice.

Immunotolerance in HEL-Tg Mice: Humoral Response

Similar to their failure to develop specific cellular response to HEL, mice of the three Tg types produced very little or no antibody against this antigen after immunization with HEL in

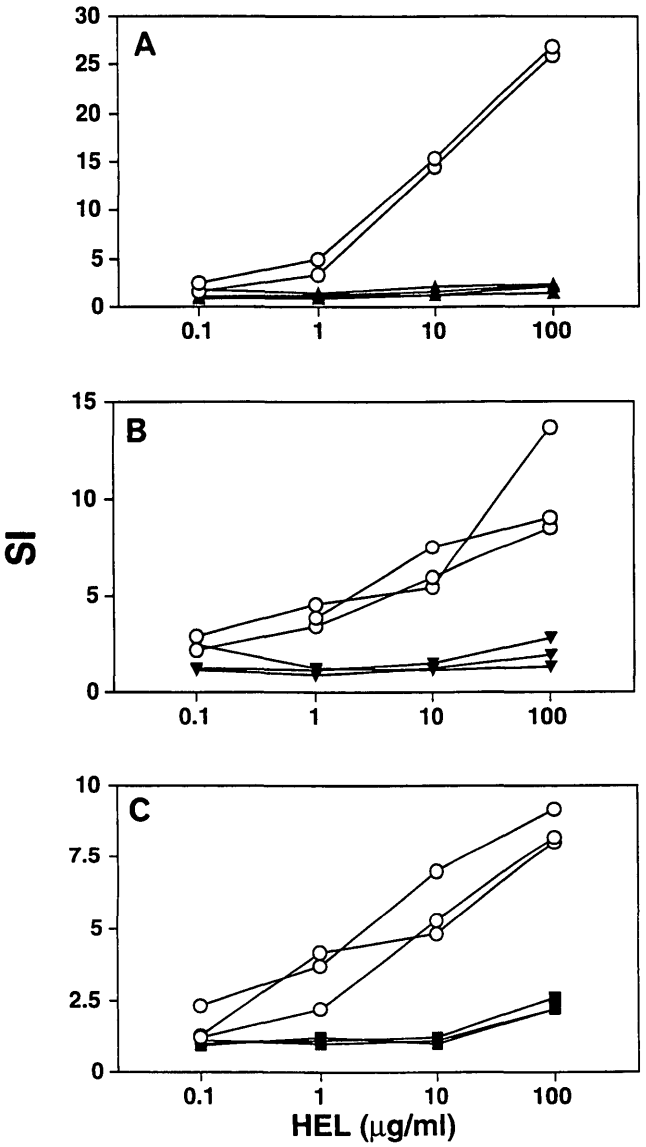

Figure 2. Hen egg lysozyme-transgenic (HEL-Tg) mice fail to develop cellular immune responses to HEL. Groups of Tg mice of the three lines and their littermate wild-type (WT) controls were immunized with HEL (25 μg) in Freund's complete adjuvant, and 14 days later their lymph node cells (**A**) or spleen cells (**B, C**) were tested for proliferative responses against the antigen at the indicated concentrations. The responses are expressed as stimulation index (SI) values of individual mice. *Open circles* denote WT, *closed symbols*, the Tg mice: (**A**) M-HEL, low copy number; (**B**) M-HEL, high copy number; (**C**) soluble form of HEL.

Freund's complete adjuvant (Fig. 4). Conversely, high levels of antibody were measured in the sera of all WT control mice. The secondary antibody used in the ELISA experiments shown in Figure 4 recognized all IgG isotypes. In addition, further analysis with isotype-specific secondary antibodies revealed that sera from immunized WT mice contained high levels of IgG1 and IgG2a but no IgM antibodies against HEL. Conversely, no HEL antibodies of any of the tested isotypes could be measured in the sera of the Tg mice (A. de Vos et al., manuscript in preparation).

HEL-Tg Mice Retain Their Immunotolerance after Enucleation

As recorded above, measurable amounts of HEL were found only in the eyes of the HEL-Tg mice, and, thus, enucleation of these animals made it possible to test the effect of removal of the peripheral source of a tolerogenic antigen on the state of immunotolerance. (Enucleation of FVB/N mice is not considered an unusually cruel procedure because these animals lose their vision at an early age due to hereditary retinal degeneration.) Groups of M-HEL high copy number and S-HEL Tg mice and their WT littermate controls were bilaterally enucleated at 4 weeks of age, and pairs of each experimental group were tested at different intervals thereafter for their immune response after immunization with the antigen in Freund's complete adjuvant. Additional control groups in each experiment consisted of sham-operated Tg mice.

Figure 5 shows the response to HEL of lymph node cells from Tg and WT mice immunized with the antigen at 6, 8, and 10 months after enucleation. The data show that cellular unresponsiveness was retained in all Tg mice tested at these time intervals after enucleation. Figure 6 summarizes the antibody levels in these groups of mice. Enucleation did not abrogate the humoral unresponsiveness in three of the five enucleated Tg mice, but a breakdown of tolerance (i.e., antibody production) was observed in two Tg mice that had been immunized with HEL 6 and 8 months after enucleation. It is noteworthy that essentially no cellular immunity to HEL was detected in the two enucleated Tg mice that produced antibody to this antigen. Antibody formation was also seen in one of two sham-enucleated Tg mice that were challenged with HEL 8 months thereafter (data not shown).

Detection of HEL mRNA in the Thymus of Tg Mice

The finding that enucleation did not abrogate the state of unresponsiveness in the Tg mice suggested that the large depot of HEL in the Tg mouse lens contributed little, if at all, to the state of tolerance in these animals. A major mechanism for tolerance induction, termed "central tolerance," is mediated by the presence of the antigen in the thymus.[11,18] As recorded above, the sensitive PCFIA could not detect HEL in the thymuses of the HEL-Tg mice. Therefore, the possible presence of trace amounts of HEL in this organ was examined with the RT-PCR, a method capable of detecting trace amounts of mRNA of the corresponding protein. The results are shown in Figure 7. No HEL mRNA was detected in the eyes or thymuses of the WT control mice. In contrast, intense responses were obtained with the eye samples of the Tg mice, and positive responses, of lower intensity, were also found in the thymus samples of all three types of Tg mice.

Discussion

Transgenic mice used in this study have provided an experimental system in which a foreign protein, HEL, is expressed in the lens, as a neo-self-antigen. No morphologic changes were observed in the eyes of Tg mice expressing S-HEL, or low levels of M-HEL, but severe ocular changes developed in the high copy number M-HEL mice (Fig. 1). In addition to total disruption of the architecture of the lens fibers and capsule, the whole eyes of these mice shrank considerably, as seen in

Table 2. Cytokine Production by TG and WT Splenocytes

Cytokine	Incubation Time (hr)	Stimulus in Culture: None (Control) WT	None (Control) Tg	HEL WT	HEL Tg	Con A WT	Con A Tg
IL-2	24	<7	<7	1274	138	>1500	>1500
IL-4	48	<5	<5	6	<5	155	138
IL-10	48	<17	<17	18	<17	823	397
IFN-γ	48	<13	<13	2645	<13	5795	4130
TGF-β	72	576	828	573	710	989	746

Tg, transgenic; WT, wild type; HEL, hen egg lysozyme; CON A, concanavalin A; IL, interleukin; IFN, interferon; TGF, transforming growth factor. Pooled splenocytes from two mice of each group were used, collected 14 days after immunization with HEL, and stimulated in culture with HEL (10 μg/ml) or Con A (1 μg/ml). The culture conditions and cytokine assays used are detailed in the Materials and Methods section. The data are presented as pg/ml.

Figure 1 and as indicated by their reduced protein content (Table 1). The mechanism for the lenticular changes is not clear, but it is assumed to be analogous to the damaging effect to the lens[19] or other tissues[20-22] by overexpression of foreign molecules such as alloantigens. Despite the severe damage, no inflammation was found in the eyes of the affected Tg mice in the present study (Fig. 1). This finding is in contrast to the intense inflammation observed in eyes in which IFN-γ,[23] IL-1,[24] or even an allogeneic major histocompatibility complex class I molecule[19] was expressed under the control of the αA-crystallin promoter. Interferon-γ and IL-1 are both proinflammatory cytokines, and the inflammation in the affected eyes is assumed to be non-antigen-specific. Conversely, the inflammation in eyes expressing the alloantigen was shown to be immune-mediated and antigen-specific.[19] The difference in inflammation development between the Tg mice expressing HEL or the alloantigen is not clear, but it could be attributed to the unique immunologic features of the major histocompatibility complex molecule. The lack of inflammation in the eyes of the HEL-Tg mice in the present study is in line with our finding of unresponsiveness to this antigen by the cellular (Fig. 2, Table 2) and humoral (Fig. 4) immune responses.

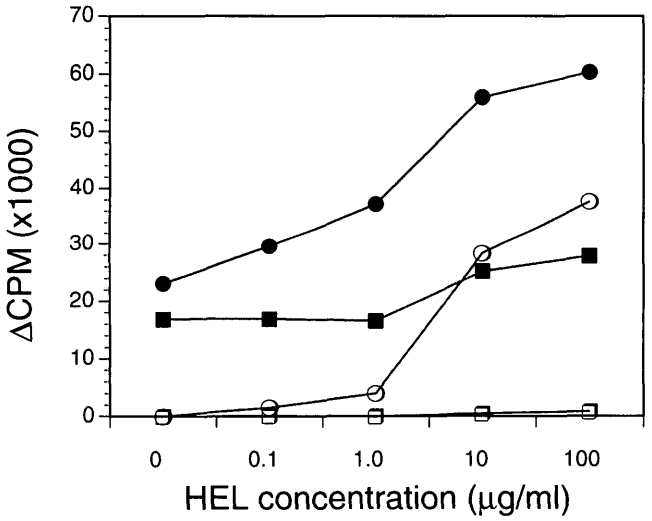

Figure 3. Effects of exogenous interleukin-2 (IL-2) on the proliferative response to hen egg lysozyme (HEL) of lymphocyte from wild-type (WT) and transgenic (Tg) mice. Lymph node cells were pooled from two mice each of WT (*circles*) and Tg with the membrane-bound form of HEL high copy number (*squares*), 14 days after immunization with HEL. Responses against HEL at the indicated concentrations were determined in the absence (*open symbols*) or presence (*closed symbols*) of IL-2 at 100 U/ml.

The lens is devoid of any vascular or lymphoid tissues and its content is sequestered from the immune system by a thick capsule. Yet, studies of recent years have revealed that some of the lens crystallins are not strictly organ-specific and that they are present in nonlenticular tissues in which they carry out various enzymatic[25] or "chaperone"[26] activities. The crystallin used here, αA, was chosen for this study because it exhibits a high level of lens-specificity; its presence in trace amounts in extralenticular tissues could be detected only by highly sensitive methods.[27,28] One nonlenticular organ with a detectable concentration of αA-crystallin is the thymus.[27,28] This observation is in line with the finding reported here that a transgene under the control of the αA-crystallin promoter is also expressed in the thymus (Fig. 7). Although the HEL mRNA transcript was readily detected in the thymuses of HEL-Tg mice, the protein itself could not be found in extracts of these thymuses with the PFCIA method, with a sensitivity threshold of 1 ng/ml. Other methods for detection of trace amounts of antigens are currently under investigation.

The finding of HEL mRNA in the thymus of the Tg mice in the present study suggests that the development of unresponsiveness to HEL in these animals could be attributed mainly to central tolerance, the mechanism by which antigen-specific T cells are eliminated in the thymus.[11,18] Actual deletion of HEL-specific lymphocytes in the thymus is difficult to detect because of the very low number of HEL-specific lymphocytes in normal animals. To overcome this hurdle in another study[29] double Tg mice were used; these mice were generated by mating our HEL-Tg mice with "3A9" Tg mice in which the majority of T cells express a receptor specific to HEL.[30] Thymuses of these double Tg mice showed a marked increase in the number of apoptotic cells, compared with thymuses of 3A9 control mice. In addition, splenocytes of the double Tg mice responded less vigorously to HEL than their 3A9 littermates,

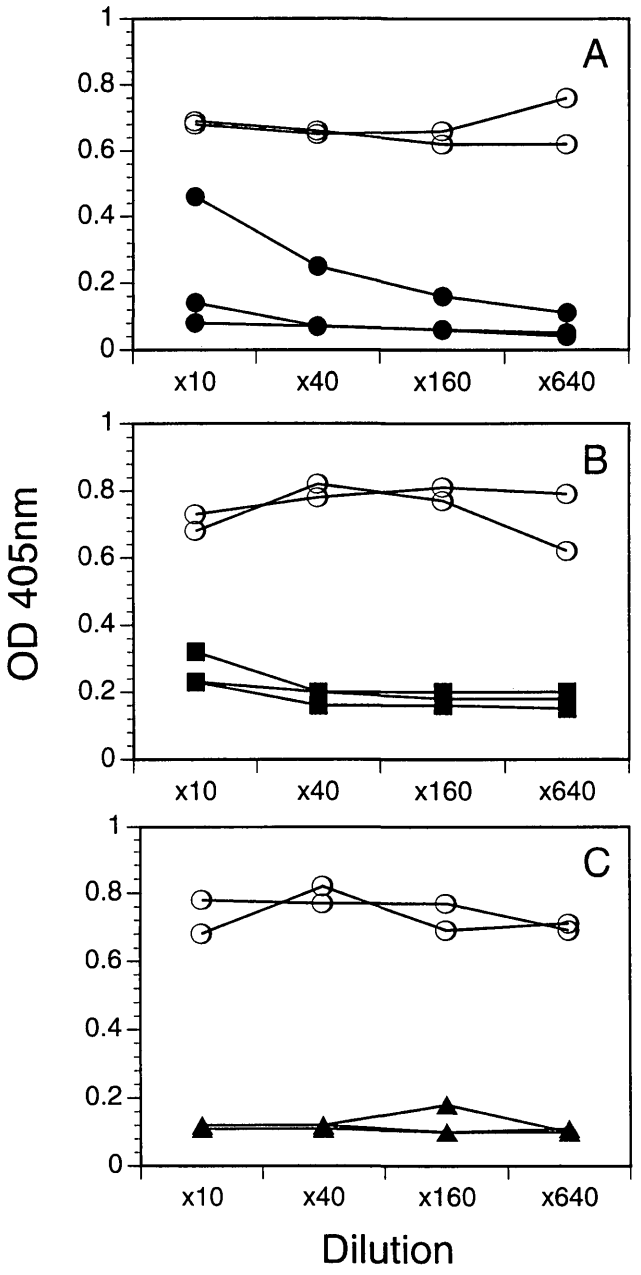

FIGURE 4. Immunotolerance of the humoral response in hen egg lysozyme-transgenic (HEL-Tg) mice. Blood samples from groups of mice as detailed in the legend for Figure 2 were collected 14 days after immunization and tested for HEL antibodies by enzyme-linked immunosorbent assay, as described in the Materials and Methods section. Shown are responses of individual mice at the indicated dilutions, expressed as optical density (OD) absorbance values at 405 nm. *Open circles,* wild-type littermate controls; *closed symbols,* Tg mice: (**A**), membrane-bound form of HEL (M-HEL) low copy number; (**B**), M-HEL, high copy number; (**C**), soluble form of HEL.

suggesting that certain populations of HEL-specific cells were deleted in the double Tg mice.[29]

The notion that central tolerance participates in the development of unresponsiveness to organ-specific neo-self-antigens in Tg mice has been supported by other studies in which immunotolerance to proteins was observed in animals that

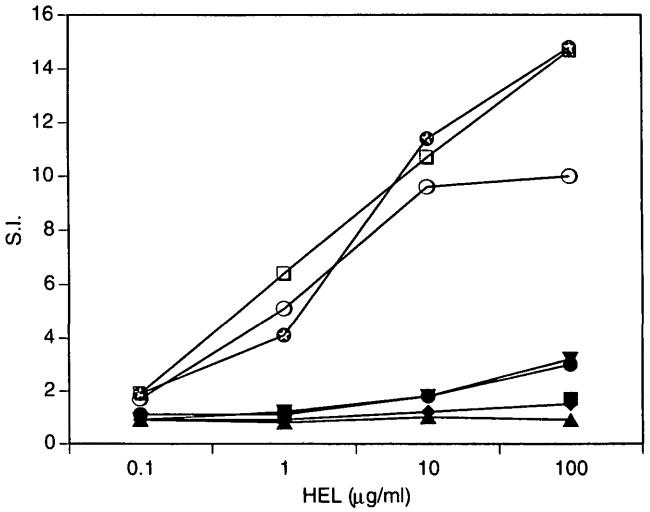

FIGURE 5. Hen egg lysozyme-transgenic (HEL-Tg) mice retain their state of cellular immunotolerance after enucleation. Tg mice and their littermate wild-type (WT) controls were challenged with HEL in Freund's complete adjuvant at different intervals after enucleation, and their lymph node cells, collected 14 days later, were tested for proliferative response against the antigen. The Tg animals (*closed symbols*) included two Tg mice with the soluble form of HEL, challenged 6 or 10 months after enucleation, and three Tg mice with the membrane-bound form of HEL (high copy number), tested 6, 8, or 10 months after enucleation. The three littermate WT controls (*open symbols*) were challenged 6, 8, or 10 months after enucleation. The data are presented as stimulation index (SI) values of each of the individual mice.

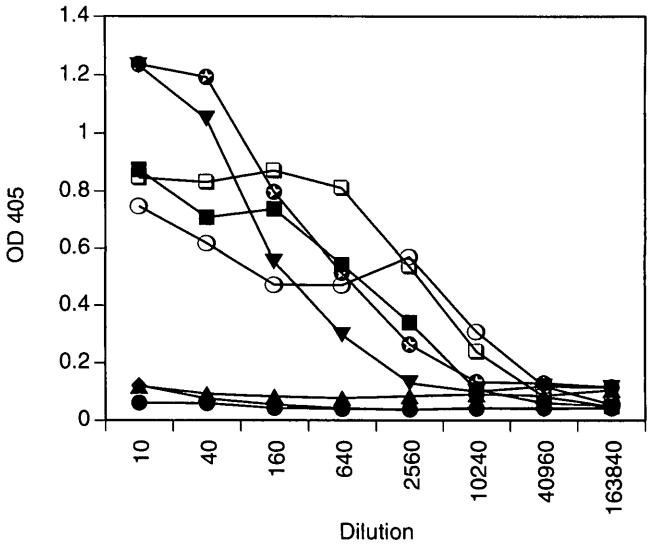

FIGURE 6. Tolerance of the humoral immunity is retained in some but not all hen egg lysozyme-transgenic (HEL-Tg) mice late after enucleation. Enzyme-linked immunosorbent assay responses of the five Tg mice and their three wild-type controls shown in Figure 5. The eight mice are identified by the same symbols used in Figure 5. The two Tg animals showing antibody activity are those with the membrane-bound form of HEL (high copy number) Tg mice, challenged 6 (▼) and 8 (■) months after enucleation.

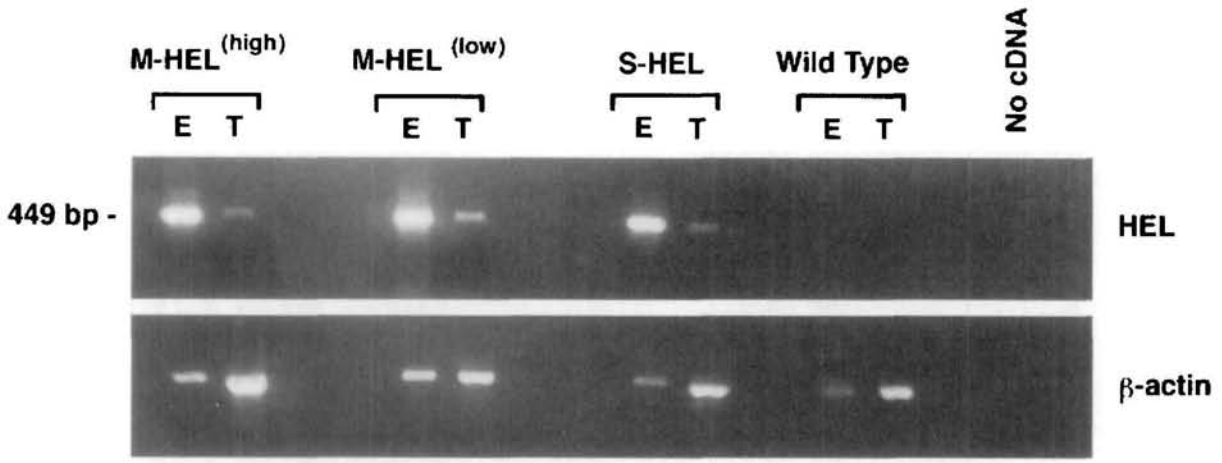

FIGURE 7. Hen egg lysozyme (HEL) mRNA in the thymus (T) and eyes (E) of transgenic (Tg) mice. The presence of specific mRNA was determined by reverse transcription-polymerase chain reaction, as detailed in the Materials and Methods section. The amplified fragments from the three Tg thymus preparations are detected, although their intensity is lower than that of the corresponding eye preparations. In contrast, only β-actin, but not HEL, is detected in the wild-type mouse organs. M-HEL, membrane-bound form of HEL; S-HEL, soluble form of HEL.

expressed the corresponding mRNA in their thymuses.[8,10] Moreover, a correlation was shown recently between thymic expression of uveitogenic retinal antigens and resistance to experimental autoimmune uveoretinitis induced by these antigens.[31]

The putative participation of central tolerance in the Tg mice used in the present study was further indicated by our finding that the state of tolerance in these animals was not abrogated by removal of the major peripheral depot of HEL via enucleation. This procedure, which did not affect the quality of life of the mice in this study (see above), made it possible to test for the first time the effect of elimination of the peripheral source of a tolerogen on the state of immunotolerance. Our data show that tolerance of humoral and cellular immune responses was retained in most enucleated mice for as long as 10 months (Figs. 5, 6), a time period sufficiently long for recovery of the immune response from a state of immunotolerance or anergy, induced by peripheral exposure to a tolerogen.[32-35]

Recent reports have attributed the unresponsiveness in various immunotolerance states to anergy.[15-17,35] However, our data do not support the involvement of anergy in the tolerance state of the HEL-Tg mice; exogenous IL-2 did not reverse the state of unresponsiveness to HEL in lymphocytes from HEL-Tg mice (Fig. 3). In addition, as mentioned above, unresponsiveness was retained in enucleated mice for time periods remarkably longer than those reported for anergy to wane in the absence of antigen (≤100 days[35]).

Our data also do not support the possibility that suppressor cells are involved in the development of unresponsiveness in the HEL-Tg mice. Splenocytes from HEL-Tg mice failed to produce significant levels of immunoregulatory cytokines, TGF-β, IL-4, and IL-10 (Table 2). In addition, we have shown elsewhere[36] that lymphocytes from WT donors sensitized against HEL produced severe ocular inflammation when adoptively transferred into HEL-Tg mice. Furthermore, spleen cultures of the recipient Tg mice were found to respond vigorously to HEL, thus suggesting that the activity of the transferred cells was not hampered by any active suppressive mechanism in the recipient Tg mice (J. C. Lai et al., unpublished data).

Cellular and humoral immune responses to HEL both were found to be diminished in most Tg mice in this study. Yet, it is conceivable that the lack of humoral response was due to the absence of helper T cells rather than to the tolerance of the B cells. This notion is in accordance with similar observations in other systems of immunotolerance[3,37] and with our observation that splenocytes from HEL-Tg mice produced very low or undetectable levels of cytokines such as IL-4 (Table 2). This notion is also in line with our finding that two Tg mice immunized with HEL 6 and 8 months after enucleation developed antibody (Fig. 6) but no detectable lymphocyte proliferative response (Fig. 5). The antibody response seen in the old Tg mice is explained by the assumption that a small fraction of helper T cells escapes the tolerogenic process in the thymus and may reach in certain mice the number needed to provide sufficient help for antibody production by the B cells. More studies are under way to examine the immune capacity of the B-cell compartment of the HEL-Tg mice used in the present study.

Our finding of tolerance against a neo-self-antigen expressed under the control of the αA-crystallin promoter sheds new light on observations made over a decade ago, that T cells of mice and rabbits are tolerant to autologous crystallins.[1,2] This tolerance against self-crystallins can now be attributed mainly to the thymic presence of these proteins. Moreover, our data suggest that peripheral tolerance has a minor role in the development and maintenance of tolerance to lens crystallins.

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