Central Immunotolerance in Transgenic Mice Expressing a Foreign Antigen under Control of the Rhodopsin Promoter

Don-II Ham,1,2,3 Stephen J. Kim,1,2,4 Jun Chen,1,2 Barbara P. Vistica,1 Robert N. Fariss,5 Robert S. Lee,6 Eric F. Wawrousek,5 Hiroshi Takase,1 Cheng-Rong Yu,1 Charles E. Egwuagu,1 Chi-Chao Chuan,1 and Igal Gery1

PURPOSE. Different conclusions have been reached in recent studies concerning the immune response or tolerance of retinal antigens. The present study was aimed to analyze the state of tolerance in Tg mice expressing hen egg lysozyme (HEL) under control of the rhodopsin promoter.

METHODS. Tg mice expressing HEL under control of the rhodopsin promoter (RhHEL-Tg) were generated and tested by conventional methods for immune responses against HEL. These Tg mice were also mated with Tg mice expressing HEL-specific receptor on their T lymphocytes and the double-Tg mice were examined for increased apoptosis in their thymi by the TUNEL assay, as well as for loss of HEL-specific T cells, by flow cytometry and proliferative response. The presence of HEL mRNA in mouse thymi was determined by RTPCR.

RESULTS. RhHEL-Tg mice developed tolerance to HEL, shown by reduced cellular and humoral responses to HEL, as well as by the failure of ocular inflammation to develop after immunization with HEL. RhHEL-Tg mice expressed HEL mRNA in their thymus, and the tolerogenic mechanism in these mice was shown to be thymic deletion of HEL-specific T cells by the following observations in the double-Tg mice: (1) increased apoptosis in their thymi, (2) remarkable reduction in the proportion of the HEL-specific T cells, and (3) loss of lymphocyte response to low concentrations of HEL.

CONCLUSIONS. Tg mice expressing HEL under control of the rhodopsin promoter develop a tolerance for the foreign antigen, apparently as a result of thymic deletion of HEL and deletion of T cells specific to this antigen. (Invest Ophthalmol Vis Sci. 2004;45:857–862) DOI:10.1167/iovs.03-1028

The immunologic repertoire is determined by the intricate selection mechanism in the thymus. Thymocytes that recognize complexes of major histocompatibility complex (MHC) and self-peptides are positively selected and later go through the negative selection process in which cells with high avidity to self-antigens are eliminated.1–4 Early notions suggested that the self-antigens that participate in the negative selection include mainly molecules that are amply expressed in the thymus5–7 or are accessible to the thymus through the circulation,7 but more recent studies have revealed that essentially all body antigens are expressed in the thymus by medullary stromal cells.5–8 For many of these antigens, however, the level of thymic expression is very low, allowing T cells with low avidity to escape negative selection. These cell escapes are thus capable of mounting an immune response toward the corresponding self-antigens, after a proper stimulation.7 Experimentally, triggering activation of T cells specific toward self-antigens is achieved by the use of adjuvants, whereas autoimmune diseases are thought usually to be facilitated by the adjuvant activity of infectious agents.8,9

Retinal antigens are sequestered from the immune system by tight junctions between endothelial cells of retinal blood vessels and between cells of the retinal pigment epithelium. This anatomic feature of the retina has been considered responsible for the self-immunogenicity of several retinal antigens, as manifested by their capacity to provoke autoimmune responses accompanied by ocular inflammation (i.e., experimental autoimmune uveitis [EAU]).10–13 Despite their anatomic sequestration from the immune system, some known uveitogenic retinal antigens were unable to trigger EAU in certain mouse strains.11 The explanation for this seemingly paradoxical observation was provided by our finding that retinal antigens are expressed in the thymus12,13 and that an inverse correlation exists between thymic expression of a retinal antigen and its capacity to induce an autoimmune response.12

Much of the information concerning immunotolerance has been collected in studies in which foreign antigens are genetically expressed under the promoter of autologous proteins. Collected data concerning the immune response against these “neo–self-antigens” are being applied to knowledge about immunity and tolerance against the native proteins.14–16 Using this approach, we analyzed the immune response and tolerance against hen egg lysozyme (HEL) expressed under control of the a-crystallin promoter.17 Two other groups have investigated the immune response against ovalbumin (OVA) (Woodward JG, et al. IOVS 2001;42:ARVO Abstract 2815) and β-galactosidase (β-gal),18–20 expressed under promoters of the retinal antigens rhodopsin (Woodward JG, et al. IOVS 2001;42:ARVO Abstract 2815) and arrestin (S-antigen).18–20 No tolerance was found in these transgenic (Tg) mice against the neo–self-antigens (Woodward JG, et al. IOVS 2001;42:ARVO Abstract 2815).18,19 A later study by Gregerson and Dou20 revealed, however, that a modification in the experimental
procedure enabled detection of tolerance in the mice expressing β-gal under control of the arrestin promoter.

The present study investigated the immune response in transgenic (Tg) mice expressing HEL under control of the rhodopsin promoter (RhHEL-Tg mice). Unlike the observations cited earlier in mice expressing foreign antigens under control of the rhodopsin promoter (Woodward JG, et al. IOVS 2001; 42:ARVO Abstract 2815), a high level of tolerance was found in the RhHEL-Tg mice. Further analysis of the RhHEL-Tg mice showed that thymic deletion of HEL-specific T cells is responsible for the state of tolerance in these animals.

**Materials and Methods**

**Mice**

RhHEL-Tg Mice. The plasmid pSK1, with HEL under transcriptional control of the mouse rhodopsin promoter, was constructed by ligation of four pieces of DNA, in a single ligation step: (1) The vector used was pKO Scrambler 902 (Lexicon Genetics/Stratagene, La Jolla, CA) which had been cut with Sall and KpnI. (2) The mouse opsin promoter fragment was isolated as a 0.5-kb KpnI/NotI fragment from plasmid MOP960-pKS3, which was kindly provided by William Hauswirth (University of Florida, Gainesville, FL). (3) The HEL gene, into which a C-terminal membrane anchor sequence had been inserted (a gift from Christopher Goodnow, Stanford University, Stanford, CA), was isolated as a 5.3kb SpeI/Sall fragment from pH2kHEL/H2k2 by complete digestion with Sall, followed by partial digestion with SpeI. (4) Oligonucleotides were synthesized both to act as a linker to join the NotI (5') end of the opsin promoter fragment to the SpeI (5') end of the HEL fragment and to introduce several unique restriction sites into this region. The sequence of the oligonucleotide pair was 5'GGCAGTTTAAACCTTAATTAACC-3' and 5'-CTAGGTTAATTGAAGTTTAAAAC-3'.

Clones selected after ligation were analyzed by restriction digestion and sequencing of selected regions, to ensure the proper construct had been generated. The 5.7-kb transgene was excised from pSK1 by digestion with Sall and Sall, gel purified, and microinjected into the pronuclei of single celled FVB/N mouse embryos to generate transgenic mice. 3A9 and Double-Tg Mice. RhHEL-Tg mice, on the FVB/N background, were mated with 3A9 mice which are on the B10.DR background (a generous gift from Mark M. Davis, Stanford University) and in which most T-lymphocytes express a T-cell receptor (TCR) background (a generous gift from Mark M. Davis, Stanford University).

**Immune Responses**

All experimental mice were immunized with 25 μg HEL (Sigma-Aldrich, 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 (0.1 mL) and the two thighs (0.05 mL) each of the mice. Cellular and humoral immune responses were measured 14 days after immunization, as described in detail elsewhere.17 Briefly, cellular responses were assessed by the lymphocyte proliferation assay, using draining lymph node cells, stimulated in culture with different concentrations of HEL. The data are recorded as the mean ΔCPM (CPM in stimulated cultures minus CPM in unstimulated controls). HEL antibody levels were measured by the enzyme-linked immunosorbent assay (ELISA), with the data presented as optical density absorbance at 405 nm.

**Histologic Development of Inflammatory Changes**

Development of ocular inflammation was analyzed in eyes of three groups of mice: RhHEL-Tg and WT mice, actively immunized with HEL, as described earlier; double-Tg mice: RhHEL-Tg mice, 7 days after adoptive transfer of polarized T-helper type 1 (Th1) cells, as described in detail elsewhere.24 The eyes were fixed, and sections were prepared through the pupillary–optic nerve plane, by conventional methods.

**Detection of HEL by Immunofluorescence**

Frozen mouse eye sections were fixed with 4% paraformaldehyde for 10 minutes, blocked by 5% normal goat serum, and stained with affinity-purified rabbit antibody against HEL, prepared in our laboratory, followed by Cy3-labeled goat anti-rabbit immunoglobulin antibody (Jackson ImmunoResearch, West Grove, PA). Cell nuclei were stained with 4',6-diamidino-2-phenylindol dihydrochloride (DAPI; Molecular Probes, Eugene, OR), added along with the labeled secondary antibody.

**Flow Cytometry Analysis**

Directly labeled antibodies specific for CD4, CD8, and Thy1.2 were purchased from BD Pharmingen (San Diego, CA). The clonotype-specific antibody, designated 1G12, that recognizes 3A9 T cells, was a generous gift from Emil Unanue (Washington University, St. Louis, MO). Single-cell suspensions were prepared and analyzed as described by Jiang and Vaccaro.25 Analysis of splenocytes and thymocytes from Tg mice was performed on a flow cytometer (FACScan; BD Biosciences). Ten thousand events were acquired in a live gate, and all un gated events were saved for later analysis.

**Reverse Transcription–Polymerase Chain Reaction**

The presence of HEL mRNA in mouse thymi was determined by the RT-PCR method, as detailed elsewhere.17 The primers used for HEL were 5'TTGGTGCTGGTGTGCTGCCCTG-3' and 5'TGCGTTTCCTCGCTGACGATC-3', and the primers for β-actin were 5'-GAGGGCGGCTTACTGACCA-3' and 5'-TCGGGCAATAGTGTAGACTTCGTCGC-3'.

**Detection of Apoptotic Cells: The TUNEL Assay**

The level of apoptosis in mouse thymi was evaluated by a cell viability assay (TACS, with the TACS 2 TdT Blue Label in situ Apoptosis Detection Kit; Trevigen, Inc., Gaithersburg, MD). Briefly, formalin-fixed, paraffin-embedded sections of mouse thymi were deparaffinized in xylene and then hydrated in graded ethanol concentrations. The tissue was digested using labeling buffer and incubated with the labeling reaction mix (Trevigen, Inc.) for 45 minutes. The labeling reaction was terminated with stop buffer (Trevigen, Inc.), and the sections were incubated with streptavidin–horseradish peroxidase (HRP) conjugate. The sections were stained (Blue Label, followed by Red Counterstain C) and then dehydrated in graded ethanol followed by xylene and mounted (Performount; Fisher Scientific, Fair Lawn, NJ).

**Results**

**Phenotype of RhHEL-Tg Mice**

Eyes of RhHEL-Tg mice exhibited normal morphology with the exception that the photoreceptor cell layer was thinner than that of the littermate WT control (Figs. 1A, 1B, respectively). Immunofluorescent staining of eyes of these mice with HEL antibody demonstrated an intense fluorescence band, restricted to the photoreceptor cell layer, as indicated by the DAPI staining of the same eye (Figs. 1C–E).
Immunotolerance to HEL in RhHEL-Tg Mice

RhHEL-Tg mice and their littermate WT controls were immunized with HEL emulsified with complete Freund’s adjuvant, and 2 weeks later all mice were tested for their humoral and cellular response against HEL, and their eyes were examined for histopathologic changes.

The humoral responses of representative mice of the two groups are shown in Figure 2A. The response of WT mice was homogenous, whereas some variability was seen among the Tg animals. All tested RhHEL-Tg mice responded with antibody levels lower than those of WT control animals.

Figure 2B summarizes a typical experiment that analyzed cellular responses to HEL of RhHEL-Tg and WT mice, measured by their lymphocyte proliferative responses. Unlike the clear reactions of the WT mice, little or no response was detected in the Tg mice.

Histologic examination of the actively immunized RhHEL-Tg mouse eyes showed no detectable inflammatory changes (not shown). It is of note, however, that adoptive transfer of as few as $3 \times 10^5$ activated HEL-specific Th1 cells induced severe inflammation in eyes of RhHEL-Tg recipient mice (described later).

Expression of HEL mRNA in Thymi of RhHEL-Tg Mice

To test the hypothesis that the state of tolerance in the RhHEL-Tg mice is attributable to central tolerance (i.e., clonal deletion in the thymus of HEL-specific T cells) we used the RT-PCR technique, to search for the expression of HEL mRNA in this organ. As shown in Figure 3, HEL transcript was readily detected in thymi of the RhHEL-Tg mice, both on the original FVB/N background, or the (FVB/N x B10.BR)F1 background. In addition, HEL mRNA was found in thymi of the double-Tg mice. The level of mRNA was similar in thymi of all three mouse lines (Fig. 3).

Partial Deletion of HEL-Specific T Cells in RhHEL-Tg x 3A9 Double-Tg Mice

To examine further the tolerogenic mechanism in RhHEL-Tg mice, we mated them to mice of the 3A9 line, in which most...
T-lymphocytes express HEL-specific TCR. The resulting double-Tg mice made it possible to demonstrate the deletion of HEL-specific cells, by the decrease in cells expressing the HEL-specific TCR and by the decline in lymphocyte response to HEL.

Figure 4 shows a representative flow cytometric analysis of spleen and thymus of double- and single-Tg 3A9 mice. In the spleen, the deletion was marked by the sharp decrease in the proportion of lymphocytes expressing the HEL-specific TCR, identified by the clonotypic antibody 1G12. Whereas this population comprised 50.8% of all spleen cells in the 3A9 control spleen, their proportion declined to only 10.7% in the double-Tg mice.

The selective deletion of thymocytes expressing the HEL-specific transgenic TCR in thymi of double-Tg mice is shown in Figure 4 by the sharp decline in the CD4 single-positive population, compared with the 3A9 control animals (from 36.5%–8.9%). This population consists of the maturing thymocytes, most of which express the transgenic HEL-specific TCR, and most of these cells were deleted in the double-Tg mice.

The deletion of HEL-specific T cells in the double-Tg mice is also demonstrated in Figure 5, which summarizes a typical experiment analyzing the lymphocyte proliferation response against HEL of spleen cells from double-Tg mice and 3A9 control animals. Double-Tg mouse cells responded much less vigorously than the 3A9 cells, and the reduced response was particularly evident in cultures stimulated with low doses of HEL. Thus, no response was detected in double-Tg mouse cultures stimulated with 0.01 or 0.1 µg/mL HEL, in contrast to vigorous responses to these concentrations by the 3A9 control cultures.

**Visualization of Thymic Deletion in Double-Tg Mice**

In view of the profound deletion of HEL-specific T cells in the double-Tg mice, we expected increased rates of apoptosis in thymi of these mice. To visualize this elevated cell death, we used the TUNEL assay, which detects apoptotic cells. As shown in Figure 6, the number of apoptotic cells (stained blue) was profoundly higher in the double-Tg mice than in the 3A9 control animals. Also of interest is the observation that most TUNEL-positive cells were located in the thymic medulla, where clonal deletion takes place.
Central Tolerance to a Retinal Neo-self Antigen

Ocular Changes in Double-Tg Mice

Because the deletion of HEL-specific T cells in the double-Tg mice was incomplete (Fig. 4), we investigated the possibility that cells that escape deletion cause inflammation in eyes of these mice, in which HEL is expressed in the retina. Histologic analysis revealed that, indeed, ocular inflammation developed in the double-Tg mice (Fig. 7A). The inflammatory changes reached their peak between the ages of 4 and 8 weeks and consisted mainly of vitritis and perivasculitis in the retina (Fig. 7A), as well as cellular infiltration throughout the retina. The histologic changes in the double-Tg mice resemble closely those induced in single-Tg RhHEL-Tg mice after adoptive transfer of small numbers of activated Th1 cells from 3A9 donors (Fig. 7B).

DISCUSSION

Data presented in this study show that RhHEL-Tg mice, expressing HEL under control of the rhodopsin promoter, exhibit development of immunotolerance toward the neo-self-antigen. The state of tolerance was demonstrated by the diminished cellular and humoral responses, as well as by the failure of ocular inflammation to develop in the RhHEL-Tg mice after immunization with HEL emulsified in complete Freund’s adjuvant. It is noteworthy, however, that the depot of HEL in the Tg mouse eyes served as a target for inflammation-inducing T cells sensitized against HEL (Fig. 7).

Similar to our previous observation in Tg mice expressing HEL under control of the αA-crystallin promoter, mRNA of the transgene was found in the present study to be expressed in the thymus of the RhHEL-Tg mice (Fig. 3). This observation suggests that the state of tolerance in the RhHEL-Tg mice is mediated mainly by clonal deletion of HEL-specific T cells in the thymus (i.e., “central tolerance”). This assumption was substantiated by experiments with double-Tg mice in which the high number of HEL-specific lymphocytes made it possible to visualize the deletion of these cells and measure their elimination from both the thymus and the periphery. Visualization of apoptosis in the thymus was achieved by the TUNEL assay (Fig. 6), and our observations are in line with those reported in other studies in which thymic deletion was examined in double-Tg mice. Similar to these reports, the increased apoptosis in the double-Tg mice localized in the thymic medulla (Fig. 6), the region where self-antigens are expressed and negative selection takes place. Flow cytometric analysis of thymocytes in the double-Tg mice revealed that the deleted population consisted mainly of single-positive CD4 (Fig. 4)—namely, the maturing 3A9 thymocytes. The selective deletion of these cells was verified by the finding that the population of cells expressing the HEL-specific TCR was drastically reduced in spleens of the double-Tg mice (Fig. 4). It is also noteworthy that the trace amounts of HEL in thymi of the double-Tg mice efficiently eliminated such a large population of HEL-specific T cells.

The selective elimination of HEL-specific T cells in the double-Tg mice was also indicated by the reduced proliferative response to HEL of splenocytes from these mice (Fig. 5). Our finding that cells of double-Tg mice failed to respond to the low HEL concentrations is in accordance with the selectivity of the deletion process toward thymocytes with high affinity toward the target antigen. Furthermore, the changes in eyes of the double-Tg mice did not differ from those in RhHEL-Tg mice after adoptive transfer of activated HEL-specific T cells from 3A9 donors (Fig. 7B). It is also noteworthy that the inflammation in these eyes is presumably facilitated by the aforementioned low levels of tissue damage indicated by the moderate hypotrophy of the photoreceptor cell layer (Fig. 1A) elicited by the expression of the transgene product.

Our finding of tolerance to the transgene product in RhHEL-Tg mice differs from the observations made by Woodward (Woodward JG, et al. IOVS 2001;42:ARVO Abstract 2815) and Gregerson et al., of no apparent tolerance to OVA (Woodward JG, et al. IOVS 2001;42:ARVO Abstract 2815) or β-gal in Tg mice expressing these antigens under control of the rhodopsin or arrestin promoters. The difference between these studies and ours could be attributable to different levels of expression of the transgene, in particular in the thymus. Indeed, no β-gal transcript was detected by Gregerson et al. in thymus of their Tg mice (Gregerson DS, personal communication, 2003). In addition, detection of immunotolerance is determined to a large extent by the immunizing stimulus. No tolerance was found in an early study by Gregerson et al. in their β-gal Tg mice when a high dose of the antigen and a powerful adjuvant (pertussis toxin) were used for immunization. In contrast, tolerance was found in a later study, in which a less potent immunization was used. Although the activity of regulatory cells was indicated in the later study, the role of thymic deletion in these Tg mice cannot be ruled out, because the immune response in the early study could be attributable to low-affinity T cells that escaped deletion and
could be stimulated by high doses of immunization \(^{32}\) and/or highly efficient antigen presentation.\(^7\)

mRNA transcripts of rhodopsin and several other ocular antigens are expressed in mammalian thymi (Refs. 12, 13 and Takase H, et al., unpublished data, 2003). Therefore, observations recorded in the current study with a neo-self-antigen expressed under the rhodopsin promoter support the notion that central tolerance is a major mechanism whereby lymphocytes specific toward these ocular antigens are eliminated, and potentially pathogenic autoimmune processes are prevented.

In summary, the data reported herein demonstrate that RhHEL-Tg mice become tolerant against the transgene product HEL. Further, the study identified the tolerogenic mechanism. HEL is expressed in the thymus of RhHEL-Tg mice, and the studies with double-Tg mice indicate that the major tolerogenic mechanism in these mice is thymic deletion of HEL-specific T cells. Moreover, it is suggested that central tolerance also prevents autoimmunity against native ocular antigens.

Acknowledgments

The authors thank Dale S. Gregerson for critical reading of the manuscript, William W. Hauswirth for the opsin promoter plasmid, Christopher C. Goodnow for the HEL plasmid, the National Eye Institute Histolah for preparing the tissue sections, and Rick Dreyfuss for digital photography.

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