Self-reactive B Cells Are Not Eliminated or Inactivated by Autoantigen Expressed on Thyroid Epithelial Cells

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Summary

Graves’ Disease results from the production of autoantibodies against receptors for thyroid stimulating hormone (TSH) on thyroid epithelial cells, and represents the prototype for numerous autoimmune diseases caused by autoantibodies that bind to organ-specific cell membrane antigens. To study how humoral tolerance is normally maintained to organ-specific membrane antigens, transgenic mice were generated selectively expressing membrane-bound hen egg lysozyme (mHEL) on the thyroid epithelium. In contrast to the deletion of autoreactive B cells triggered by systemic mHEL (Hartley, S.B., J. Crosbie, R. Brink, A.B. Kantor, A. Basten, and C.C. Goodnow. 1991. Nature. 353:765–769), selective expression of mHEL autoantigen on thyroid cells did not trigger elimination or inactivation of circulating HEL-reactive B cells. These results provide evidence that tolerance is not actively acquired to organ-specific antigens in the preimmune B cell repertoire, underscoring the importance of maintaining tolerance to such antigens by other mechanisms. The role of an intact endothelial barrier in sequestering organ-specific antigens from circulating preimmune B cells is discussed.

Autoantibodies directed against molecules that are unique to the surface of cells in the parenchyma of discrete organs underlie the pathogenesis of a variety of organ-specific autoimmune diseases (1). For example, production of autoantibodies against the thyroid-stimulating hormone (TSH) receptor cause the thyrotoxicosis of Graves’ Disease, and anti-thyroid peroxidase antibody in Graves’ and Hashimoto’s thyroiditis is thought to inhibit thyroid function and promote complement deposition and thyroid destruction (2). Similarly, antibodies to the acetylcholine receptor interfere with neuromuscular synaptic transmission in Myasthenia Gravis (3), antibodies to epithelial cell cadherins cause cell detachment in bullous pemphigoid and pemphigus vulgaris (4), and antibodies against type IV collagen lead to Goodpasture’s Disease (5).

Recent studies have established that one bulwark blocking the production of autoantibodies against self antigens that are displayed in the bloodstream or on the surface of circulating cells is the active elimination or inactivation of self-reactive B cells from the preimmune repertoire (6–10). This process operates for autoantibodies even with very low affinity to membrane-bound self-antigen (11, 12). By contrast, for organ-specific antigens, the relative ease with which autoimmunity can be induced by immunization has long suggested that B cell inactivation or elimination is either reversed by immunization with potent adjuvants (13, 14) or that the B cell repertoire is simply not censored to these types of antigens. Ig gene transgenic mice have been used to test these alternatives for one organ-specific antigen in mice expressing a Kb histocompatibility antigen on hepatocytes (MT-Kb) (15). In this case, the Kb-specific B cells were clonally deleted from the preimmune repertoire. The Kb antigen in these mice was controlled by the metallothionein promoter, however, which is active in many tissues, including bone marrow–derived cells (16), so that B cell deletion to MT-Kb may have reflected low-level systemic antigen expression.

To determine whether autoreactive B cells are normally eliminated or inactivated to self-antigen displayed selectively on the surface of parenchymal cells in specific organs, we have modified a systemically expressed membrane hen egg lysozyme (HEL) transgene (9) to direct expression exclusively to the thyroid epithelium. The thyroid gland was chosen as a prototype for several reasons. First, the thyroid is a highly vascularized tissue, so that antigens expressed uniquely in the thyroid should not be physically sequestered from the circulating immune system (17), as may oc-

Abbreviations used in this paper: cGG, chicken gamma globulin; HEL, hen egg lysozyme; mHEL, membrane-bound HEL; nPP, disodium p-nitrophenyl phosphate; pBS, Bluescript plasmid; rTg, rat thyroglobulin; sHEL, soluble HEL.
cur in the eye or brain. Second, the thyroid epithelium has considerable regenerative potential and is physically well characterized. Third, animal models of experimental autoimmune thyroiditis (EAT) have suggested that humoral tolerance to thyroid-specific antigens is easily broken or nonexistent (18, 14). Tolerance to thyroid antigens also seems fragile in humans, as ~40% of women 20 yr or over show thyroid inflammation at autopsy (19). Finally, autoantibodies directed against thyroid-specific antigens have a well-established role in the pathogenesis of autoimmune thyroid disease (2). By using Ig transgenic mice that have previously shown elimination (9) or inactivation (6) of B cells to systemic antigens, we find that, by contrast, thyroid-specific membrane-bound HEL (mHEL) induces no detectable censoring of the preimmune B cell repertoire.

Materials and Methods

Creation of TLK Transgenic Mouse Lines. The TLK gene construct uses the rat thyroglobulin (rTG) promoter to direct expression of mHEL on the thyroid epithelium. The rTG promoter was excised from pR-geo (20) as a 3.3-kb HindIII-EcoRI partial digest fragment in which the HindIII overhang was filled in by Klenow. This was then subcloned into an EcoRI-X ba digest of the Bluescript plasmid (pBS) in which the Xba overhang was also blunt-ended, thus regenerating the Xba site. The resulting rTG promoter was excised using Xba partial digest with ClaI complete digest. A mHEL construct containing HEL fused to the H-2 Kb transmembrane region has been previously described (9). Partial Xba and complete ClaI digestion of this construct allowed the insertion of the rTG promoter upstream of the mHEL gene. For oocyte microinjection, the TLK construct was excised with CiaI and KpnI, purified, and microinjected into C57BL/6 eggs as described (6). Two transgenic founders (TLK-1, TLK-2) were obtained and maintained on the B6 background.

All animals were maintained on the C57BL/6J (B6) genetic background and were used between 6 and 20 wk of age. To obtain B6 mice expressing the Ly5a allele, B6-Ly5a congenic mice were bred to B6 (Ly5b) to generate Ly5b/heterozygous animals.

RT/PCR Analysis. Approximately 50–100-mm² sections from various mouse tissues were removed and immediately minced in 4 M guanidine thiocyanate solution (Fluka, Switzerland) containing 0.1 M 2-mercaptoethanol using a 5–ml glass and teflon tissue grinder (PGC Scientific, Gathersburg, MD). Total RNA was obtained using standard procedures. cDNA was prepared from 500 ng total RNA using oligo-dT primer, and PCR was performed from 1/10 of the cDNA reaction with both actin and HEL primers in the same reaction mixture.

Immunohistochemistry on Frozen Tissue Sections. Thyroid was removed from C022 euthanized animals and placed into plastic containers (Cryomold; M ilees, Elkhart, IN), immediately covered in O.C.T. (M ilees), slowly frozen by floating on liquid nitrogen, and stored at −80°C until cut. 6-10-μm sections were cut on a microtome at −20°C and collected onto PTFE-coated slides (Hendley-Exton, Essex, Essex, England). Sections were dried overnight, fixed for 10 min in acetone (at 4°C), and stained in a humid chamber at room temperature with biotinylated primary antibody (H-YHEL-9-biotin) as described (21). Avidin-alkaline phosphatase second stage (Sigma Chemical Co., St. Louis, MO) and Fast Red/Naphthol AS-MX substrate (Sigma) were used to visualize specific staining as described (21). Sections were counterstained in hematoxylin and mounted in Crystal/Mount (Biomedra Corporation, Foster City, CA).

Immunization. HEL and chicken gamma globulin (cGG) were obtained from Sigma. HEL-cGG protein conjugate was the kind gift of Dr. Kevan Shokat (Howard Hughes Medical Institute, Stanford University, Stanford, CA). Animals were injected i.p. with either 100 μg of HEL or 25 μg HEL-cGG emulsified in 200 μl PBS containing RIBI adjuvant (Ribi Immunocor, Rexsearch, Hamilton, MT) as described in the manufacturer. Animals were either killed on day 14 or 28 after immunization, or boosted on day 28 with the same amount of HEL or HEL-cGG in RIBI. Boosted animals were killed and analyzed 7 d later.

Anti-HEL Enzyme-Linked Immunosorbent Assay. HEL (Sigma) was purified on an ion exchange column, dissolved in carbonate buffer, pH 9.2, and used to coat 96-well flat-bottom plates (Flow Laboratories, Inc., Mclean, VA). The plates were blocked for 3 h at 37°C with 10 mg/ml BSA (Pentex, Kankakee, IL). Serum and standards diluted in 1 mg/ml BSA in PBS, pH 7.5, were then applied to duplicate wells. Bound anti-HEL IgG was developed with goat anti-mouse IgG (Fc specific) conjugated to alkaline phosphatase (catalog no. A-2429, Sigma), and bound anti-HEL IgM was developed with goat anti-mouse IgM conjugated to alkaline phosphatase (catalog no. A-2429, Sigma). Disodium 4-nitrophenyl phosphate (NPP) substrate (Sigma) was then applied and plates read in a Molecular Devices (Menlo Park, CA) Emax plate reader at 405 nm. The concentrations of anti-HEL IgG were determined relative to a standard curve of anti-HEL IgG a from a transfectedoma. Anti-HEL IgG values were calculated in units by reference to a standard curve of H-YHEL-8 anti-HEL IgG a mAb.

Flow Cytometry. Spleen, lymph node, and thymus cells were isolated by passing through a metal sieve, washed in media containing 2% FCS/RPMI 1640, and counted with a hemocytometer. Thyrocytes were prepared by mincing two thyroid lobes with a scalpel blade in serum-free HBSS, washed once in serum-free HBSS in a 15-ml Falcon tube, and 150 μl of 20 mg/ml collagenase (Boehringer Mannheim, Mannheim, Germany) was added to the pellet. The thyroid lobes were incubated for 10 min at 37°C with intermittent tapping, washed twice with 2% FCS/RPMI 1640 media, and resuspended in 200 μl media. 100 μl was used for FACS® staining. Cells were analyzed by flow cytometry on an FACS® Facsym® (Becton–Dickinson, Mountain View, CA) with FACS® Desk software (Beckman Center Shared FACS Facility). The following antigens and antibodies were used: B220, RA3-6B2-PE (Caltag, South San Francisco, CA) and RA3-6B2-Fluorescein (FITC; Caltag); Ly5a, AS20-FITC and AS20-biotin; IgM α, R S3.1-PE and R S3.1-FITC; IgD α, AMS9.1-FITC; HEL-binding, HEL (Sigma) and H-YHEL-9-biotin, or H-YHEL conjugated to cyochrome. Biotinylated reagents were detected with streptavidin-Cychrome (Pharmingen, San Diego, CA). All antibody stains were performed on 5 × 106 cells using standard procedures as described (6, 22).

A adoptive Transfer. Unfractionated spleen cells from Ly5b/h transgenic mice were isolated as above and analyzed by flow cytometry to determine frequency of HEL-binding transgenic B
cells. Spleen cells containing $10^7$ HEL-binding transgenic B cells were mixed with $10^7$ spleen cells from Ly5^a/b nontransgenic mice and injected into recipients via the lateral tail vein. Recipient animals were sacrificed 5 or 10 d after transfer and analyzed as above by flow cytometry.

**Thyroid Blood Flow Calculations.** Calculations for blood flow through mouse thyroid were based on available values from human (17, 22, 23). As measured fractional cardiac output to various tissues in the mouse (skin, kidney, heart, muscle) are within 50% of those measured in human (23), and since fraction of body weight represented by the thyroid is also similar (0.03% versus 0.02%), we assume that fractional cardiac output to thyroid in the mouse will also be similar to that in human. Given a fractional cardiac output value of either 1 or 2.8% to the thyroid, we can calculate the number of circuits (n) between systemic and pulmonary circulations required before 50 or 99.9% of circulating lymphocytes have traveled through the thyroid gland. Assuming mouse blood volume is ~2.3 ml and C.O. is 16 ml (23), n will be given in units of ~9-s intervals. If $x = \% \text{C.O.} \to \text{thyroid}$, then

$$(1 - x)^n = 0.5 \text{ (time for 50\% cells through gland)}$$

or

$$(1 - x)^n = 0.001 \text{ (time for 99.9\% cells through gland)}$$

Solve n (in quantities of 15 s) for:

$$(1 - x)^n = \frac{\log(0.5)}{\log(1 - x)} \text{ or } \frac{\log(0.001)}{\log(1 - x)}$$

**Results and Discussion**

Transgenic mice expressing membrane-bound HEL on the thyroid epithelium were produced by microinjecting C57BL/6 eggs with a gene construct containing 3.3 kb of the rat thyroglobulin promoter (24) linked to the membrane HEL gene (Fig. 1a). Two transgenic lines, TLK-1 and TLK-2, were established, and mice from either line exhibited normal size, body weight, and fecundity, indicating that the transgene did not induce overt thyroid insufficiency. RT-PCR analysis of various tissues revealed that most or all mHEL RNA expression is restricted to the thy-

![Figure 1](http://rupress.org/jem/article-pdf/186/12/2005/1111770/97-1154.pdf)

TLK-2 thyroids. 10-μm frozen sections of one thyroid lobe from each line was stained using a biotinylated antilysozyme mAb, streptavidin-alkaline phosphatase secondary stage, and Fast red substrate (red). Hematoxylin (blue) was used for counter-staining. D) Flow cytometric analysis of thyrocytes from nontransgenic (thin line) or TLK-2 transgenic (thick line) mice stained for cell surface HEL. The percent positive cells is indicated.
B Cell Tolerance to Thyroid Surface Antigen

Immunohistochemical analysis of thyroid gland from both lines revealed abundant HEL expression on many follicular epithelial cells (Fig. 1c). Western blotting showed that the membrane-bound form of HEL was made in large quantities in the TLK-2 thyroid (25), and FACS® staining of thyrocytes confirmed surface expression of HEL (Fig. 1d). There was no evidence of inflammation in any of the mice, and follicular architecture was normal except in a fraction of older mice from the TLK-2 line (data not shown). The latter animals displayed moderately enlarged thyroids, with follicles that appeared hyperplastic and consisted of cuboidal epithelium, although there was no evidence for inflammation. Because the TLK-2 line expresses higher levels of HEL than TLK-1, the cytopathology present in a fraction of older TLK-2 animals may be a direct effect of transgene expression, as described previously (26–28), but appears not to affect acquisition of immune tolerance (see below).

To determine whether or not thyroid mHEL expression resulted in tolerance to HEL, TLK-1 or TLK-2 transgenic mice and nontransgenic littermates were immunized with HEL. Nontransgenic mice mounted normal primary and secondary IgG responses to HEL, whereas very little antibody was produced in transgenic mice (Fig. 2a). Thus, thyroid mHEL animals actively acquired tolerance to HEL either at the B or T cell level, or both. To focus on the B cell repertoire, any T cell tolerance to HEL that may have been acquired was bypassed by immunizing with HEL antigen covalently linked to a foreign carrier, chicken gamma globulin (HEL-cGG). After HEL-cGG immunization, HEL-specific IgG was produced in equal titers in nontransgenic and transgenic mice from both TLK lines (Fig. 2b). This result therefore indicated either that preimmune B cells were not censored to HEL or that provision of potent T cell help to the cGG carrier was able to override B cell elimination or inactivation.

The status of circulating HEL-specific B cells in the preimmune repertoire was directly examined by crossing TLK-1 and TLK-2 thyroid mHEL transgenic mice with Ig transgenic mice that express high-affinity HEL-specific IgM and IgD on 90% of circulating B cells (6). When Ig × TLK double-transgenic mice were compared with littermates carrying only the Ig transgene, no differences in B cell phenotype (Fig. 3a) or number could be detected in spleen (Fig. 3a and b), lymph node, or bone marrow (not shown). This contrasted with the change in B cell phenotype in mice expressing circulating sHEL antigen (Fig. 3a) (6) or the deletion of HEL-binding B cells in mice expressing mHEL in a systemic distribution (9). In addition, serum levels of transgenic anti-HEL IgMa were not reduced in Ig-transgenic animals expressing thyroid mHEL compared with Ig-transgenic mice lacking HEL (Fig. 3c), unlike the inhibition of anti-HEL autoantibody secretion in mice expressing HEL systemically (6, 9).

The large number of HEL-specific B cells that are constantly produced in Ig-transgenic mice may in principle have obscured deletion of small, physiological frequencies of autoreactive B cells by encounter of HEL on the thyroid. To test this possibility, small numbers of naive, mature HEL-specific Ig-transgenic cells were introduced into the bloodstream of unirradiated nontransgenic or TLK transgenic mice and allowed to circulate for 5 or 10 d. To provide an internal standard for the transfer and ensure detection of subtle losses of HEL-specific B cells, Ly5a-marked nontransgenic spleen cells were coinjected with the Ly5b Ig-transgenic cells. The behavior of these Ly5a nontransgenic B cells should not be affected by the presence or absence of HEL antigen. Despite the presence of only trace numbers of circulating HEL-reactive B cells, their number and ratio to nontransgenic standard B cells was not significantly different in TLK recipients with high expression of mHEL in the thyroid gland compared to non-transgenic recipients lacking HEL autoantigen (Fig 4a).

The maintenance of tolerance to organ-specific self anti-
gens is vital for the avoidance of autoimmune diseases such as Type I Diabetes, Hashimoto’s thyroiditis, Graves’ Disease, and Myasthenia Gravis. In this report, we demonstrate that expression of HEL specifically on the thyroid epithelium resulted in actively acquired tolerance that could not be broken by immunization with HEL in adjuvant. Humoral tolerance to thyroid HEL did not result, however, from the elimination or functional inactivation of high affinity autoreactive B cells from the circulating preimmune repertoire, in contrast to tolerance to systemic HEL (6). Thus, humoral tolerance to thyroid mHEL could easily be broken by immunization with a conjugate that linked self-HEL epitopes to a foreign carrier (Fig. 2 b). Crosses or cell transfer with anti-HEL Ig-transgenic mice clearly established that thyroid mHEL did not eliminate or inactivate HEL-reactive B cells at appreciable efficiency (Figs. 3 and 4).

Two factors may in principle explain the failure of actively acquired B cell tolerance to thyroid-specific surface HEL antigen: (a) very few B cells may pass through the thyroid circulatory bed; or (b) circulating B cells may be kept separate from HEL on thyroid epithelium by the basement membrane and vascular endothelium. The former is unlikely based on the pattern of thyroid blood flow and proportion of cardiac output received in humans (17; Table 1), which predicts that all circulating B cells should traffic through the thyroid many times in the 5- or 10-d period studied in the experiments of Fig. 4 (Table 1 and see Materials and Methods for calculations). On the other hand, the continuous endothelium lining the capillary beds surrounding thyroid follicles is likely to create a physical barrier against preimmune B cells contacting thyroid mHEL. In the absence of inflammation, the thyroid capillary endothelium lacks the addressins to allow trafficking of preimmune B cells into the organ parenchyma (29). By contrast, elimination of circulating autoreactive B cells to liver-specific H-2Kb membrane autoantigen (15) may reflect the fact that hepatocytes do not have such a barrier between their plasma membrane and circulating cells (30).

Figure 3. Lack of B cell tolerance in TLK transgenic mice crossed to anti-lysozyme Ig transgenic mice. (A) Two-color FACS® analysis of spleen cells from nontransgenic, Ig transgenic, and Ig × TLK double-transgenic mice. HEL-binding B cells were identified by staining cells with an excess of unlabeled HEL before secondary staining with a complementary biotinylated anti-HEL monoclonal antibody. RS-3.1 (anti-IgM*) co-staining reveals that the HEL-binding cells are all expressing transgenic (a-allotype), rather than endogenous (b-allotype), receptor. For comparison, spleen cells from Ig × ML-5 double-transgenic mice expressing soluble HEL systemically are shown, illustrating the phenotypic changes occurring in anergic self-reactive B cells (6). (B) Number of anti-HEL splenic B cells in Ig transgenic and Ig × TLK double-transgenic mice, measured as in A. (C) Serum levels of anti-HEL IgMa (transgenic) antibody. No reduction in spontaneous secretion of transgenic anti-lysozyme antibody is seen in Ig × TLK double-transgenic mice compared to Ig-transgenic littermates.
across the endothelium (32, 33). Nonetheless, bound IgG may not be sufficient to induce complement and recruit inflammatory cells on its own due to low concentrations of complement and inflammatory cells in healthy tissue and to the thyroid's intrinsic ability to inhibit complement activation via CD46, CD55, and CD59 (34, 35). Indeed, different EAT models have not shown a correlation of inflammation to anti-thyroglobulin, anti-TPO, or anti-TSH receptor antibody (18, 36–38).

Failure of active B cell tolerance may occur for tissue-specific antigens in many different parenchymal organ systems with comparable vascular barriers to the thyroid. Indeed, preimmune B cells are also not censored in transgenic mice expressing mHEL in pancreatic islet beta cells (ILK-3 transgenic line, data not shown). These results imply that secretion of pathogenic autoantibodies in autoimmune diseases such as Graves' disease, Myasthenia Gravis, Bullous Pemphigoid, and Goodpasture's Disease is not controlled at the level of preimmune B cells. These autoantibodies may arise when T cell tolerance is bypassed by immunological challenges with cross-reactive foreign antigens or with self antigen that became coupled to a foreign carrier (Fig. 2, a and b), or when T cell tolerance breaks down. It will be important in future work to determine the extent to which thyroid-specific antigens trigger active regulation of self-reactive T cells or censoring of B cells at later stages of the immune response, for example in local germinal centers or when the thyroid becomes inflamed and B cells are attracted into the organ.

Table 1. Summary of Thyroid Circulatory Parameters

|                         | Human | Mouse |
|-------------------------|-------|-------|
| Cardiac output (C.O.)   | 5 liter/min | 16.0 ml/min |
| Thyroid weight          | 20 g  | 4.5 mg |
| Body weight             | 70 kg | 25 g  |
| Thyroid blood flow      | 5 ml/g/min | ???   |
| % body weight (thyroid) | 0.03% | 0.02% |
| % C.O. to thyroid       | 2.8%  | ???   |

Table 2. Calculation Times for 50 or 99.9% Circulating Lymphocytes to Pass through Thyroid Gland

| % C.O. to thyroid (x) | (1 − x) | time 50% | time 99.9% |
|-----------------------|---------|----------|------------|
|                        |         | min      |            |
| 2.8%                  | 97.2%   | 3.66     | 36.49      |
| 1.0%                  | 99.0%   | 10.34    | 103.10     |
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