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Enhancing or Suppressive Effects of Antibodies on Processing of a Pathogenic T Cell Epitope in Thyroglobulin

Yang Dai,* Karen A. Carayanniotis,* Petros Eliades,† Peggy Lymberi,† Philip Shepherd,‡ Yi-chi M. Kong,§ and George Carayanniotis*‡

Thyroglobulin (Tg)-specific Abs occur commonly in thyroid disease, but it is not clear to what extent they affect Tg processing and presentation to T cells. Here we show that the generation of the nondominant pathogenic Tg epitope (2549–2560), containing thyroxine (T4) at position 2553 (T4(2553)), is augmented by Tg-specific IgG mAbs that facilitate FcR-mediated internalization of Tg. However, other mAbs of the same (IgG1) subclass enhanced Tg uptake by APC but had no effect on the generation of this peptide. Treatment of APC with chloroquine or glutaraldehyde abrogated enhanced generation of T4(2553). The boosting effect was selective, since the enhancing mAbs did not facilitate generation of the neighboring cryptic (2495–2511) peptide, which is also pathogenic in mice. When Tg was simultaneously complexed to a mAb reactive with T4(2553) and to a mixture of boosting mAbs, the presentation of this epitope was totally suppressed. These results suggest that Tg-specific Abs alter Tg processing and may boost or suppress the presentation of nondominant pathogenic determinants during the course of disease. The Journal of Immunology, 1999, 162: 6987–6992.

Major histocompatibility complex class II-restricted T cells generally respond to a limited number of immunodominant epitopes in foreign or self antigenic proteins (1). Ag-bound Abs are known to influence this response pattern and facilitate generation of subdominant or cryptic T cell epitopes (2) in several ways. First, Abs may increase the efficiency of Ag capture by FcR+ professional APC by 103- to 104-fold (3), leading to increased Ag delivery in the processing compartment (4) and production of cryptic epitopes over a critical threshold level required for T cell activation. Second, Abs may modulate Ag processing by preventing T cell epitopes from loading onto MHC class II molecules regardless of whether these epitopes overlap with peptides bound by the Ab combining site (5–7). This may suppress the generation of dominant determinants while augmenting the presentation of nondominant epitopes. Third, internalization of immune complexes (ICs) may up-regulate MHC class II synthesis or expression of adhesion and costimulatory molecules on APC, increasing their stimulatory capacity. These pleiotropic effects may act synergistically, mediating a spreading of the immune responses to epitopes distinct from those that initiated it. The above concepts are based on experimental evidence obtained mostly with bacterial Ags, but they have profound implications in T cell-mediated autoimmune diseases. Capture of autoantigen by slg on B cells, or autoantigen-Ab complexes by FcR+ dendritic cells or macrophages, may activate autoreactive T cells (8, 9) or mediate the spreading of the T cell response from dominant to cryptic epitopes (10).

In this study, we have used thyroglobulin (Tg) as a model Ag to examine whether processing of Tg-Ab complexes would generate pathogenic but nondominant T cell epitopes. Tg is a 660-kDa homodimer, the largest autoantigen known, that causes experimental autoimmune thyroiditis (EAT), a T cell-mediated disease considered as a model for Hashimoto’s thyroiditis (HT) in humans (11). Two observations render Tg an ideal choice for this study. First, none of the known pathogenic T cell epitopes in Tg has been classified as dominant in EAT studies (12). Second, a large percentage of patients with HT, and almost invariably all animals developing EAT, have circulating Tg-specific Abs. It remains unclear whether such Abs play a role in promoting the generation of cryptic pathogenic epitopes. We have developed T cell hybridoma clones against two pathogenic MHC class II-binding peptides at the C-terminal end of Tg: the subdominant peptide (2549–2560), which is derived from processing of Tg in vivo but not in vitro (13, 14), and the cryptic peptide (2495–2511), which is not generated following processing of intact Tg either in vivo or in vitro (15). The aim of the study was to examine whether mAbs bound to Tg would interfere with Tg uptake or processing by APC and promote the generation of these two peptides.

Materials and Methods

Antigens

The 17-mer Tg peptide (2495–2511) Ac-GLINRAKVKQEESQG-NH2 (15) and the 12-mer Tg peptide (2549–2560), NH2-STDDT4ASFRRAL-NH2 (containing thyroxine (T4) at amino acid position 2553 (T4(2553))) (16) were synthesized and purified as previously described. Both peptide sequences are part of the mouse Tg sequence (17). Tg was purified from frozen thyroid glands of outbred ICR mice (Bioproducts for Science, Indianapolis, IN) by passing thyroid homogenates through a Sepharose CL–4B column (15). The fractions of peak II were pooled, concentrated to

Footnotes

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2 Address correspondence and reprint requests to Dr. George Carayanniotis, Faculty of Medicine, Memorial University of Newfoundland, St. John’s, NF Canada, A1B 3V6. E-mail address: gcarayan@morgan.ucs.mun.ca

3 Abbreviations used in this paper: IC, immune complex; Tg, thyroglobulin; EAT, experimental autoimmune thyroiditis; HT, Hashimoto’s thyroiditis; T4, thyroxine; Td(2553), peptide (2549–2560) with T4 at amino acid position 2553; LNC, lymph node cell.

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3–5 mg/ml PBS, filter sterilized, and stored at −20°C until use. In all assays, Tg concentrations are expressed as the molarity of the monomeric form (330 kDa).

**Culture medium and cell lines**

All assays were performed in DMEM (Life Technologies, Burlington, Ontario, Canada) supplemented with 10% FBS (Bioproducts for Science), 10 mM HEPES buffer, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies) and 5 × 10⁻⁵ M 2-ME (Sigma, St. Louis, MO). The TA3 cell line, produced by fusion of B cells from CAF, mice with the M12.4.1 BALB/c B lymphoma (18), was kindly provided by L. Glomcher (Harvard Medical School, Cambridge, MA). TA3 cells are known to express Aαβ, Eαβ, Kαβ, IgG, k-chain, and FcγR molecules, while they are negative for IgM, IgD, Thy-1.2, and K k (18, 19). The IL-2-dependent CTL-L.2 (20) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The T cell hybridoma 3.47 clone was generated following a modified method of Perkins et al. (21).

Briefly, lymph node cells (LNC) from CBA/J mice, immunized with mouse Tg, were further stimulated in vitro with the same Ag and fused with BW5147 αβ cells (22) (a kind gift of P. Marrack, Howard Hughes Medical Institute, Denver, CO) using polyethylene glycol 1500 (Boehringer Mannheim, Indianapolis, IN). Screening and cloning were performed as previously described (23). The 3.47 clone is CD4+ by FACS and is Aα restricted as assessed by mAb inhibition of activation (data not shown). The 8F9 T cell hybrid clone (CD4+ and Eγ restricted) was similarly produced from LNC of B10.BR mice primed and boosted with the pathogenic Tg peptide (2495–2511).

**Purification and characterization of mAbs**

The previously characterized 5D2, 3B3, 2A4, and 3C4 mAbs (24) were produced from hybridomas formed between spleen cells from BALB/c mice immunized with human Tg and mouse myeloma NSI/Ag 4.1 cells. They were purified from culture supernatants by affinity chromatography on protein G-Sepharose 4 Fast Flow columns (Pharmacia, Baie d’Urfe, Montreal, Canada). Fixation of TA3 was performed by suspending 4 × 10⁶ TA3 cells in 0.5 ml of culture medium (10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies) and 5 × 10⁻⁵ M 2-ME (Sigma, St. Louis, MO)). To monitor IC uptake by APC, Tg was 125I labeled using iodogen (Pierce, Rockford, IL) and 125I-labeled sodium (DuPont Canada, Mississauga, ON) according to the manufacturer’s protocol. All free 125I-labeled sodium was removed by extensive dialysis before IC formation. Potential cross-reactivity for pairs of mAbs was assessed by RIA based on competitive inhibition. Briefly, wells of microtiter polystyrene plates (Dynatech, Chantilly, VA) were coated by a Tg-specific mAb (Sigma A5420) (data not shown). Fragmentation of 3C4 to F(ab) 2 was done using preactivated papain as described (25). The purity of the F(ab) 2 preparation was confirmed by SDS-PAGE, and its binding to Tg was verified by ELISA. The rat anti-mouse FcR mAb 2.4G2 (IgG2b) was purified by affinity chromatography on protein G-Sepharose from supernatants of HB 197 cells (ATCC).

**ELISA and RIA**

The mAbs were tested for reactivity against mouse Tg or Tg peptides by ELISA (15) using Fc or Fab-specific alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) as the second Ab. Absorbance at 405 nm was measured using a microplate reader (Vmax, Molecular Devices, Menlo Park, CA). To monitor IC uptake by APC, Tg was 125I labeled using iodogen (Pierce, Rockford, IL) and 125I-labeled sodium (DuPont Canada, Mississauga, ON) according to the manufacturer’s protocol. All free 125I-labeled sodium was removed by extensive dialysis before IC formation. Potential cross-reactivity for pairs of mAbs was assessed by RIA based on competitive inhibition. Briefly, wells of microtiter polystyrene plates (Dynatech, Chantilly, VA) were coated by a Tg-specific mAb (30 μg/ml), and 125I-labeled Tg (240,000 cpm/μg) was allowed to bind to the wells in the presence of increasing amounts of a second Tg-specific mAb. After washing and drying, the wells were cut and counted in a gamma counter (Wallac, Gaithersburg, MD).

**T cell activation assay**

ICs of Tg and mAbs were formed in DMEM in triplicate wells of 96-well culture plates. One hour later, 10⁵ TA3 and 10⁵ T cells were added in a total volume of 200 μl/well. Following a 24-h incubation, 100 μl of supernatant was removed and stored at −20°C for assay of IL-2 release, as measured by the proliferation of the CTL-L.2 line using [3H]thymidine (DuPont Canada). Fixation of TA3 was performed by suspending 4 × 10⁶ cells/ml in 0.05% glutaraldehyde. After 30 s, the reaction was stopped by the addition of an equal volume of 0.2 M glycine.

**Results**

**Uptake of Tg-mAb ICs by APC is blocked by an anti-FcR mAb**

Initial testing by ELISA confirmed that the five mAbs, 5D2, 3C4, 3B3, 2A4, and 55H8, which were raised against human Tg, cross-reacted significantly with mouse Tg (Fig. 1A). Some differences in binding were, however, observed, with 5D2 and 55H8 mAbs exhibiting a 10- to 15-fold better binding to mouse Tg than the others. To directly assess mAb-mediated uptake of Tg by APC, we pulsed TA3 cells for 6 h with 200 nM of 125I-labeled Tg either free or complexed with equimolar amounts of various mAbs was used to pulse 10⁵ TA3 cells in 0.5 ml of culture medium for 6 h at 37°C. The anti-FcR mAb 2.4G2 was used at 10 μg/ml. Cell-bound radioactivity was measured after extensive washing. Each value is the mean of triplicate wells. Similar results were obtained in two independent experiments.

**FIGURE 1.** Tg-specific mAbs promote IC uptake by APC. A. Reactivity of purified mAbs 55H8, 5D2, 3C4, 3B3, and 2A4 against mouse Tg (1 μg/ml), as assessed by ELISA. Each point represents the mean value of triplicate wells. B. Enhanced uptake of Tg-Ab complexes by TA3 is blocked by anti-FcR mAb. 125I-labeled Tg (at a final concentration of 200 nM, 170,000 cpm/μg) either free or complexed with equimolar amounts of various mAbs was used to pulse 10⁵ TA3 cells in 0.5 ml of culture medium for 6 h at 37°C. The anti-FcR mAb 2.4G2 was used at 10 μg/ml. Cell-bound radioactivity was measured after extensive washing. Each value is the mean of triplicate wells. Similar results were obtained in two independent experiments.

To follow the processing of Tg-mAb complexes, we monitored the generation of two neighboring pathogenic peptides, T4(2553) and T4(2495–2511), using TA3 as APC. The first peptide was serendipitously found to activate 3.47, an Aα-restricted T cell hybridoma clone that was, nevertheless, unreactive to equimolar concentrations of intact Tg (Fig. 2A). Processing of Tg bound to 5D2 and
3C4 boosted presentation of the T4(2553) epitope that was now detectable at 5–10 nM (Fig. 2B), whereas the 2A4-Tg complex was 10- to 20-fold less efficient. On the other hand, binding of 3B3 and 55H8 mAbs to Tg had no augmenting effect on the generation of T4(2553), despite the fact that they enhanced Tg uptake by TA3. The boosting effect did not correlate either with the mAb IgG subclass (e.g., 3C4, 3B3, and 55H8 are all IgG1) or with the relative mAb binding to Tg (e.g., 5D2 and 3C4 augment equally well the generation of T4(2553)).

The second epitope (2495–2511) was recognized by 8F9, an Eα-restricted T cell hybrid clone. 8F9 is similar to 3.47 in sensitivity for free peptide detection (1–2-nM range) and in unresponsiveness to intact Tg (2–200-nM range) (Fig. 2C). However, unlike 3.47, 8F9 did not respond to TA3 cocultured with any of the above Tg-mAb complexes (Fig. 2D). Since 5D2 and 3C4 lowered the threshold for presentation of the T4(2553) peptide without boosting the presentation of the second determinant localized 38 aa upstream, the boosting effect of these mAbs could not be attributed solely to enhanced Tg uptake by the complexes. These results suggested that 5D2 and 3C4 exerted a selective augmenting effect by interfering with the loading of the T4(2553) epitope on Aβ molecules during Tg processing.

**5D2 and 3C4 mAbs interfere with Tg processing**

To exclude the possibility that the 5D2 and 3C4 mAbs exerted signaling effects on TA3 that would generally augment Ag presentation, we titrated Tg or T4(2553) in the presence or absence of a fixed amount of mAb followed by the addition of TA3 and 3.47 T cells. 3.47 was again activated only after processing of Tg-mAb complexes, not intact Tg (Fig. 3A). In contrast, 5D2 and 3C4 showed no boosting effect on T4(2553) presentation when free peptide was used as Ag (Fig. 3B), which argues against non-Ag-specific signaling effects of these mAbs on TA3. Addition of chloroquine in the culture abrogated the enhancing effect of 5D2 or 3C4 on the presentation of the T4(2553) peptide following Tg processing but did not influence the activation of 3.47 cells by TA3 pulsed with free peptide (Fig. 3C). Finally, glutaraldehyde-fixed TA3 cells pulsed with Tg-5D2 or Tg-3C4 complexes did not activate the 3.47 clone but retained the capacity to present free T4(2553) peptide to the same T cells (Fig. 3D). These data indicated that 5D2 and 3C4 exert their effects during Tg processing either by facilitating the generation of the T4(2553) peptide or by augmenting the loading of this epitope on Aβ molecules.

**FcR-mediated uptake of Tg-Ab complexes is necessary for the generation of T4(2553) peptide in TA3 cells**

Following digestion with preactivated papain, we obtained F(ab')2 fragments from the 3C4 mAb. In ELISA, these F(ab')2 fragments retained Tg-binding activity similar to that of intact 3C4 (data not shown). It was subsequently observed, however, that TA3 pulsed with Tg bound to 3C4 F(ab')2 did not activate the 3.47 clone (Fig. 4A). Also, addition of increasing amounts of FcR-specific mAb in TA3 cultures completely blocked the activation of 3.47 mediated by 5D2-Tg or 3C4-Tg complexes, whereas the same treatment had no effect on the stimulation of 3.47 by free T4(2553) peptide (Fig. 4B). These results demonstrated that the FcR-mediated uptake of Tg-Ab complexes by TA3 is a necessary step for the formation of the T4(2553) epitope.

**The 55H8 mAb suppresses T4(2553) generation during processing of Tg-mAb complexes**

We subsequently screened by ELISA all mAbs for potential reactivity against the two Tg peptides. The 55H8 mAb was reactive with the T4(2553) epitope (Fig. 5A), whereas the 5D2 mAb bound specifically to (2495–2511) (Fig. 5B). None of the other mAbs.

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**FIGURE 2.** Selective generation of the pathogenic Tg epitope T4(2553) following processing of Tg-Ab complexes. IL-2 release following activation of 3.47 (A and B) or 8F9 (C and D) T cell hybrid clones by the Ags shown in the presence of TA3 cells, as assessed by the proliferation of the IL-2-dependent CTLL line. Each point represents the mean of triplicate wells. Similar data were obtained in three independent assays. Neither T cell hybrid responded to equimolar concentrations of free mAbs (data not shown).

**FIGURE 3.** Enhanced presentation of T4(2553), mediated by 3C4 and 5D2 bound to Tg, requires an intracellular Tg processing step. Results show the activation of the T4(2553)-specific, IL-2-secreting 3.47 T cell hybrid clone. A and B. Enhanced presentation does not result from a non-specific effect of mAbs on TA3. Shown is 3.47 T cell activation following titration of Tg (A) or T4(2553) peptide (B) in the presence or absence of a fixed amount (200 nM) of 5D2 or 3C4 mAbs and TA3. Each point represents the mean IL-2 release of triplicate wells. C. Chloroquine (67 nM) abrogates the enhanced generation of T4(2553) following processing of Ab-bound Tg (50 nM) in TA3 cells but has no effect on the presentation of equimolar amounts of free peptide. D. Fixation of TA3 by 0.05% glutaraldehyde abrogates generation of T4(2553) from Tg-Ab complexes but has no effect on the presentation of free peptide.
reacted with either of these two peptides. This observation explained the lack of augmenting function by 55H8 and further suggested that 55H8 might prevent loading of T4(2553) on Aκ molecules during Tg processing, possibly due to peptide sequestration, as predicted by the T cell:B cell reciprocity hypothesis (5, 6). To test this, equimolar amounts of 55H8, Tg, and a boosting mAb (3C4 or 5D2) were mixed in wells of microtiter plates in the presence of TA3 and 3.47 T cells. The 3B3 mAb, which cannot augment presentation of T4(2553) (Fig. 2B) and does not bind to it (Fig. 5A), was used as control. It was found that 55H8 abolished the augmenting effects of 3C4 or 5D2 mAbs on the generation of this determinant, whereas the control 3B3 mAb was ineffective (Fig. 6A). This dominant suppressive effect was also prevalent when all three potentiating mAbs, 5D2, 3C4, and 2A4, were cocultured with Tg and 55H8 (Fig. 6B). 55H8 did not inhibit binding of Tg to 55H8 in a competitive inhibition RIA. Similarly, the lack of suppressive effects by 3B3 could not be explained by abrogation of binding due to interference; none of the mAbs inhibited binding of radiolabeled Tg to 3B3 (Fig. 5D).

Discussion

This study demonstrates the presence of Tg-specific Abs that enhance or suppress the generation of a nondominant pathogenic T cell epitope. Enhancement of peptide presentation results from a combined effect of Abs on increased Tg uptake and altered Tg processing. FcR-mediated endocytosis of IC by APC is a necessary step in this process because of the blocking effects of the FcR-specific mAb and the failure of 3C4 F(ab’)2 fragments to augment T4(2553) presentation. On the other hand, increased uptake of IC by APC is not sufficient to allow generation of the same epitope, because the 3B3 or 55H8 mAbs enhanced uptake of IC by TA3 cells but did not augment presentation of the T4(2553) peptide. Altered processing may occur if enhancing mAbs, such as 5D2 and 3C4, remain tightly bound to Tg at the endosomal acidic pH, stabilizing a domain that contains this epitope and facilitating the transport of this determinant in a peptide-loading compartment (26).

The failure of 3C4 to boost generation of the cryptic (2495–2511) peptide provides a clear example of a qualitative effect on Tg processing. This soluble mAb, piggybacked on Tg, enhances Tg capture by TA3, but this does not lower the activation threshold for (2495–2511) to allow its detection by the 8F9 clone. How can the 3C4 mAb that binds to a monomeric Tg subunit of 2748 aa residues have such contrasting effects on the generation of two epitopes that are only 38 aa apart? 3C4 does not bind to (2495–2511), and thus a plausible interpretation would be that endosomal proteases trim the 3C4-bound fragment of Tg and remove the (2495–2511) peptide because it is localized beyond the boundary of 3C4 protection. Thus, it is conceivable that mAbs with different
specificities from the ones used here will be found to promote presentation of (2495–2511).

The TA3 line has been produced after fusion of B cells with a B cell lymphoma, and its inefficiency in the processing of intact Tg is paradoxical, since the rate of fluid-phase pinocytosis in B cell tumors is 10- to 60-fold higher than that of B cells (27). In earlier studies, Hutchings et al. (28) demonstrated that B cells from Tg-primed mice presented low amounts of Tg (1 µg/ml) to a T cell hybridoma clone that was subsequently found to react with (2549–2560) (29). Our data and those findings demonstrate that Tg-reactive IgG either in soluble form or as slg on B cells can mediate enhanced Tg uptake by receptor-mediated endocytosis and modulate Tg processing to enhance the generation of this pathogenic epitope. Interestingly, the above data (28) also imply that B-cell clones bearing the 55H8 specificity do not occur with high frequency in Tg-primed spleen cells, but this interpretation requires caution, since the 55H8 mAb was elicited after challenge with human Tg, whereas Hutchings et al. (28) used a pool of mouse, dog, and rat Tg for immunizations.

Other investigators have similarly reported that Abs bound to foreign (30–32) or self (33) Ags can modulate presentation of T cell determinants. In apparent contrast to our data, Fab fragments did mediate enhanced presentation of peptide Ag in some studies (31), but the APC were EBV-transformed, tetanus toxin-specific B cell clones that constitutively internalized IC via their slg. When B-cell lymphomas were used as APC, use of F(ab')2 fragments in IC did not improve Ag presentation (30), and FcγR-specific Abs blocked T cell activation (30, 33), suggesting FcR-mediated augmentation of Ag uptake and presentation. Other FcR-expressing APC such as macrophages or dendritic cells might similarly process Tg-Ab complexes to generate the peptides studied here, but the outcome of the response is difficult to predict, given that distinct sets of proteases may be active in different APC (34). Assays based on adoptive transfer of Ag-specific LNC have clearly shown that the nondominant pathogenic (2495–2511) and/or T4(2553) peptides are generated within the mouse (13, 15, 16) or rat (35) thyroid, possibly via processing of Tg or Tg fragments by intrathyroidal dendritic cells (36).

Ab-mediated suppression of Tg peptide generation is exemplified by the 55H8 mAb that binds to T4(2553) on Tg and may sequester this epitope from loading onto A2 molecules. This interpretation is in agreement with the T cell:B cell reciprocity hypothesis (5, 6), according to which the slg on B cells, or soluble Ab bound on Ag, can at times negatively steer Ag processing because the part of the Ag within the Ab-combining site is protected from proteolysis and prevented from subsequent loading onto MHC class II molecules. A corollary of this hypothesis would be that soluble 55H8 piggybacked on Tg would exert a dominant suppressive effect on the generation of this pathogenic epitope. This is fully supported by our data; when mixtures of potentiating mAbs and 55H8 were incubated with Tg, suppression was dominant. Suppression could not be attributed to 55H8 blockade of the T4(2553)-A2 complex, because the same S2-Tg-55H8 IC preparations did not affect presentation of free peptide on TA3 (our unpublished data). A reciprocal relationship is also highlighted by the S2 mAb and the (2495–2511)-specific 8F9 clone, which recognize overlapping determinants within (2495–2511); this peptide cannot be generated during processing of S2-Tg complexes in TA3, because the T and B cell epitopes physically overlap. To our knowledge, our findings provide the first examples that directly support the T cell:B cell reciprocity hypothesis in autoimmune disease.

The present study reveals an immunoregulatory role for Tg-specific IgG Abs that are quite frequent in HT and include all four subclasses (37–39). The chronicity of the disease favors generation of high affinity IgG1 and IgG4 Abs (40), and it has been suggested that rising titers of high affinity IgG1 anti-Tg may be indicative of impending hypothyroidism (40). As extrapolated from our data, such high affinity autoantibodies may have a neutral, suppressive, or augmenting effect on the generation of pathogenic Tg T cell determinants, depending on the epitopes they recognize and the genetic background of the individual. In some cases, their net effect could be exacerbation of disease and the spreading of the autoimmune response to nondominant T cell epitopes (41). At present, a more direct testing of this hypothesis with Tg-reactive human CD4+ T cell clones is not feasible, because the epitopes they recognize remain unknown. In addition, we do not know whether Tg-specific human autoantibodies in HT recognize linear or conformational determinants (37) and where these determinants are precisely localized. Several studies suggest, however, that autoreactive IgG in the serum of HT patients binds to a limited number of Tg epitopes (24, 39, 42–44), including an immunodominant central region (aa 1149–1250) (45). Autoreactive, Tg-specific Abs may be induced by foreign Ags via molecular mimicry and gradually promote the generation of nondominant pathogenic T cell epitopes, as suggested in other systems (8). Our data are not incompatible with this concept and support the notion that Tg-reactive Abs may play a much more complex role in the regulation of thyroid disease than previously anticipated.

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