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Antigenic determinants of human thyroglobulin differentiated using antigen fragments

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Summary. Human thyroglobulin (Tg) was digested with V8 protease and the fragments separated by high performance liquid chromatography (HPLC). The antigenic relationship of the fragments was investigated using mouse monoclonal antibodies to human Tg. The binding of Hashimoto's disease autoantibodies to the fragments was measured by radioimmunoassay. This demonstrated that a minority of the patients recognize an epitope on Tg which others do not. The epitopes identified by the autoantibodies were substantially destroyed in the smaller fragments tested, but these smaller fragments were more efficient stimulators of Tg-specific T-cell lines than the larger fragments which carry the antibody binding determinants. This suggests that the parts of the Tg molecule which stimulate autoimmune B cells differ from those which stimulate T cells.

INTRODUCTION

The breakdown of tolerance which occurs in autoimmune thyroiditis has been examined in mice and shown to be dependent on the action of T cells (Vladutiu & Rose, 1975). Furthermore, the ability to produce an autoantibody response is controlled by Ir genes at the I-A subregion (Vladutiu & Zalenski, 1981), although genes at the K and D loci also influence the incidence of thyroiditis. It is possible that T cells and B cells recognize different determinants on the Tg molecule in a way analogous to the expression of epitopes on lysozyme, where determinants stimulating T cells or B cells can be isolated on different fragments of the molecule (Benjamin *et al.*, 1984). In spite of the evidence that the T cells control autoreactivity in autoimmune thyroiditis, it has not been possible, so far, to determine which part of the Tg molecule reacts with T cells and to compare this with the parts reacting with antibody. This problem has been compounded by the complexity of the Tg molecule itself.

Analysis of the B-cell response (antibody) to thyroglobulin in humans shows that autoantibodies recognize a much more limited range of epitopes than xenoantibodies (Roitt, Campbell & Doniach, 1958; Mates & Shulman, 1967; Nye, Pontes de Carvalho & Roitt, 1980). Two approaches have been made towards determining how the epitopes are expressed on Tg. The first has been to treat the molecule physically, or to fragment it enzymatically and analyse residual activity of antisera against the fractions (Stylos & Rose, 1969, 1977; Salabè, Davoli & Fontana, 1973).

Abbreviations: BSA, bovine serum albumin; DEAE, diethyl amino ethyl; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; RIA, radioimmunoassay; Tg, thyroglobulin.

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The second approach has been to use monoclonal antibodies to human or mouse thyroglobulin to investigate the number of independent epitopes or antigenic domains expressed on the molecule (Rose *et al.*, 1982; Ruf *et al.*, 1983).

In this paper, we describe how we have used enzymatic digestion of human thyroglobulin with V8 protease to generate a series of antigenically active fragments, and resolved them using HPLC. With the use of monoclonal antibodies, we have determined how the fragments are related to each other antigenically. In order to determine whether autoantibodies and T cells react with different parts of the molecule, we have compared the pattern of antibody binding to the different fragments in RIA with their ability to stimulate T-cell lines responsive to a cross-reacting determinant on mouse and human Tg. During the course of this study, we have noted heterogeneity in the epitope recognition pattern of different Hashimoto's disease sera, and we believe that the use of antigen fragments such as those used here will permit a better delineation of the autoantigenic determinants on thyroglobulin, both with respect to antibody production and T-cell stimulation.

MATERIALS AND METHODS

Thyroglobulin digestion

Human thyroglobulin was prepared by differential ammonium sulphate precipitation (Derrien, Michel & Roche, 1948) and gel filtration on Sepharose 6B. The purified thyroglobulin ran as a single peak on HPLC G3000. Thyroglobulin was digested by adding 1:50 w/w staphylococcal V8 protease (Miles Biochemicals, Slough, Berks) to Tg dissolved at a concentration of 50 mg/ml in PBS. The mixture was incubated for 1·5 hr at 37°, and then urea was added to the digest to give a final concentration of 6 M. This step was to stop the digestion and to dissociate the fragments produced. The digest was then resolved using HPLC on a TSK G3000 column equilibrated with 6 M urea in phosphate buffer. Flow rate (0·04 ml/min) was optimal for molecules of approximately 40,000 MW. HPLC produced approximately 15 fractions with molecular weights between 10,000 and 460,000. Molecular weights were derived by interpolation from standards run under the same conditions (Tg, IgG, BSA, ovalbumin, DNAse, cytochrome *c*). The fractions were pooled as indicated in Fig. 1. The urea was removed by dialysis against PBS containing 0·05% azide and the fragments were stored at 4°.

Ion exchange chromatography was carried out on an HPLC TSK 545 DEAE column equilibrated in 0·01 M phosphate, pH 7·0. The fractions were applied in this buffer and eluted with an exponential gradient of 0–0·3 M NaCl at pH 7·0.

Radioimmunoassay

RIA plates (Dynatech, Alexandria, VA) were sensitized with 5 μg/ml of human Tg or Tg fragments in PBS overnight at 4°. The plates were then blocked with 4 mg/ml BSA in PBS for 30 min and washed three times with PBS.

Serum samples and supernatants containing mono-

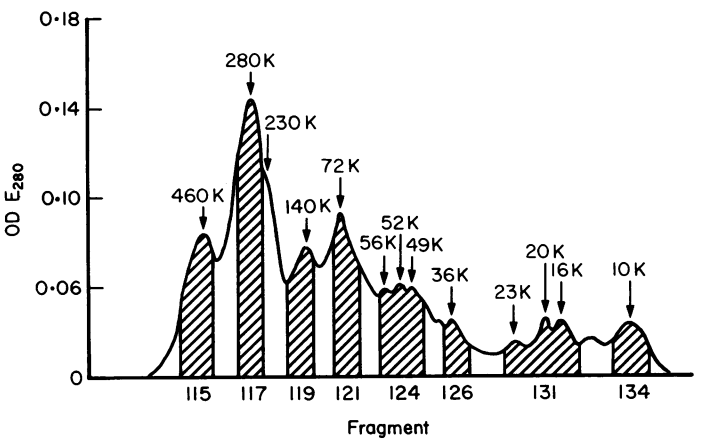

Figure 1. Human thyroglobulin digested with V8 protease was resolved by HPLC. The fractions were pooled in seven groups (115–134). Molecular weights of the different fractions derived by interpolation from standards are shown above the peaks.

clonal antibodies were diluted in RIA diluent (4 mg/ml BSA, 0·05% Tween 20 in PBS) and 100 μl aliquots were applied to each well for 2 hr at 37°. The plates were washed three times in PBS and bound antibody was detected using either 300 ng/ml [^{125}I] protein A (4·0 mCi/mg) for human antibodies or 500 ng/ml [^{125}I] sheep anti-mouse F(ab′)$_2$ (2·0 mCi/mg) for mouse antibodies. The plates were incubated for 2 hr at 37° with the labelled conjugates, washed three times with PBS (for mouse antibody) or 0·14 M phosphate, pH 8·0 (for human antibody), before being cut and counted. Rabbit anti-human Tg prepared by repeated immunization with purified Tg in CFA was used as a positive control in all experiments, and was detected using protein A conjugate.

Stimulation of Tg-specific cell lines

The preparation of Tg-specific cell lines is described fully elsewhere (Champion *et al.*, 1985). Briefly, lymph node or spleen cells from CBA/Ca mice immunized with mouse Tg in CFA were propagated *in vitro* by repeated stimulation with mouse Tg and syngeneic irradiated spleen cells in the absence of added growth factors. After 3 months, IL-2 containing supernatants from Con A stimulated rat spleen cells were added to the culture medium for the Tg-specific cell lines.

Stimulation of thyroglobulin-specific or PPD-specific (control) T-cell lines was assayed by culturing 2×10^4 cells with 5×10^5 irradiated (2000 rads) spleen cells in the presence of PPD, human Tg or Tg fragments. Tg fragments were tested at five different concentrations (0·5–20 μg/ml). After 2 days, cultures were pulsed with 0·5 mCi [^{125}I]deoxyuridine and harvested onto glass fibre discs after a further 16 hr incubation. Results are expressed as mean incorporation of label ± SEM of triplicate cultures.

RESULTS

Digestion of human Tg

Human thyroglobulin was digested with *Staphylococcus aureus* strain V8 protease, and the resulting digest was separated into fractions of different molecular weights by HPLC on a TSK G3000 column (Fig. 1). The digestion produced a variety of fragments with molecular weights between 10,000 and 460,000. Undigested Tg run under identical conditions gave a single protein peak. Under the stated conditions, the digestion was reproducible but variations in the time of digestion produced marked changes in the proportions of each fragment, and reduction of disulphide bonds (with 2-mercaptoethanol or dithiothreitol) produced different fragments. Different batches of thyroglobulin produced slight variations from the pattern shown in Fig. 1. It was noted that the digestion procedure is not complete at 1·5 hr, since extending the digestion procedure led to further breakdown of the larger fragments and subsequently some aggregation of the digestion products.

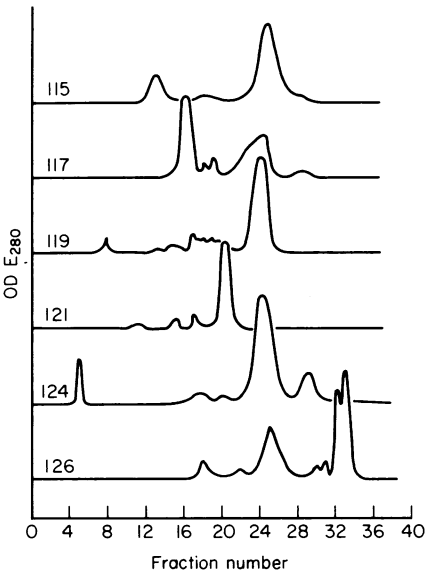

Figure 2. HPLC G3000 fractions 115–126 were resolved by HPLC DEAE column chromatography in a gradient of 0–0·3 M NaCl at pH 7·0. The chromatograms indicate a degree of heterogeneity within the HPLC G3000 peaks.

Fragments 115–126 were further analysed by HPLC DEAE ion exchange chromatography. The chromatogram profiles (Fig. 2) show that there are several peptides discernible in each fraction, although, in most cases, one peptide constitutes the majority of the material. Some of the heterogeneity is probably derived from the heterogeneity of the undigested Tg but, with smaller fractions such as 126, there appear to be several peptides with similar molecular weights. SDS PAGE analysis of unreduced fragments showed that there was no overlap between the peaks pooled from the HPLC G3000 column but, when analysed by reduced SDS PAGE, different fragments shared some peptides, indicating that the smaller fragments are breakdown products of the larger fragments. The

relationship between the fragments was further examined using antibodies.

Antigenic characterization of fragments

All the fragments except for the smallest (134) retained some degree of antigenicity, since they were able to bind rabbit anti-human Tg in radioimmunoassay. RIA plates coated with the largest fragments (115, 117, 119 and 121) bound anti-Tg 80–90% as efficiently as Tg-coated plates, while the smaller fragments, 124, 126 and 131 bound 50%, 13% and 15%, respectively.

In order to examine the antigenic relationship of the fragments to each other, the binding of monoclonal anti-human Tg antibodies to each fragment was measured in RIA using tissue culture supernatants containing hybridoma antibodies. The results are shown in Table 1. The studies using the monoclonal antibodies 1D6 and 3B3 show that there are some determinants on undigested Tg which are absent from even the largest fragments. There must be more than one of this type of epitope since 3B3 binds to mouse Tg as well as human Tg, while 1D6 does not. The fragments 115–126 all bind monoclonals 2B4, 2A4 and 4E7, indicating that these fragments are successive breakdown products of each other, or at least overlap to the extent of shared epitopes. The fragments cannot be separate parts of the Tg molecule unless the recognized epitope recurred in different parts of the molecule. This conclusion is supported by the SDS PAGE data mentioned above. The binding studies with monoclonals 1D4 and anti-T4 indicate that the two smallest fragments have lost some of their epitopes, but the determinant recognized by 1D4 cannot be the thyroxine residues on the molecule since it does not react with mouse Tg and the latter is known to express determinants reacting with anti-T4.

Table 1. Reactivity of monoclonal antibodies to thyroglobulin fragments

Monoclonal	Antigen*							
	HuTg†	115‡	117	119	121	124	126	MTg§
1D6	+	−	−	−	−	−	−	−
2B4	+	+	+	+	+	+	+	−
1D4	+	+	+	+	+	−	−	−
3B3	+	−	−	−	−	−	−	+
2A4	+	+	+	+	+	+	+	+
4E7	+	+	+	+	+	+	+	+
Anti-T4¶	+	+	+	+	+	−	−	+

* Binding of monoclonal anti-human thyroglobulin antibodies to antigens measured in radioimmunoassay. Supernatants giving more than double the background counts with an irrelevant supernatant are scored +.

† HuTg = human thyroglobulin.

‡ 115–126 = human thyroglobulin fragments.

§ MTg = mouse thyroglobulin.

¶ Monoclonal antibody to thyroxine (T4).

Recognition of Tg epitopes by autoantibodies

The reactivity of Hashimoto's disease sera autoantibodies with Tg epitopes was analysed in RIA using plates sensitized with the Tg fragments. The Hashimoto sera were diluted at 1/50 and the bound antibodies were detected with [^{125}I]protein A. The great majority of anti-Tg antibodies in Hashimoto sera are IgG and are therefore detectable with protein A. Binding activity for the fragments is shown in Fig. 3. The larger fragments (115–121) bound antibody from the majority of patients, but there was considerably less binding activity to the smaller fragments (124–131). There was no detectable binding to 134, either with the Hashimoto sera or with the + control rabbit anti-Tg; therefore, this is either due to 134 lacking epitopes recognized by any of the sera, or the plate was inadequately sensitized.

When the degree of binding activity of the different sera against the fragments was analysed, it was found that there was a high degree of correlation between binding activity to 117 and 119 (Fig. 4b), indicating that Hashimoto patients recognize an epitope(s) expressed on both these fragments. Correlation between binding to other fragment pairs was less marked (115:121, 117:121, 119:121, 115:117, 115:119, 121:124). Since it was shown above that these fragments are successive breakdown products of the Tg molecule, this implies that the epitopes recognized by the Hashimoto sera are partly degraded during the digestion, but that there is no loss of epitope expression associated with the loss of peptides in the conversion of 117 into 119.

It was noted that, when comparing the counts of the different Hashimoto sera against the fragments, a proportion reacted relatively strongly against 117 and 119 by comparison with (for example) 115 or the whole Tg molecule. This is illustrated in Fig. 4a which clearly shows that reactivity of the sera against 117 has a bimodal distribution whereas, for example, reactivity to 121 is unimodal. This implies that there is an epitope expressed on 117 (and 119), but not on 115 or

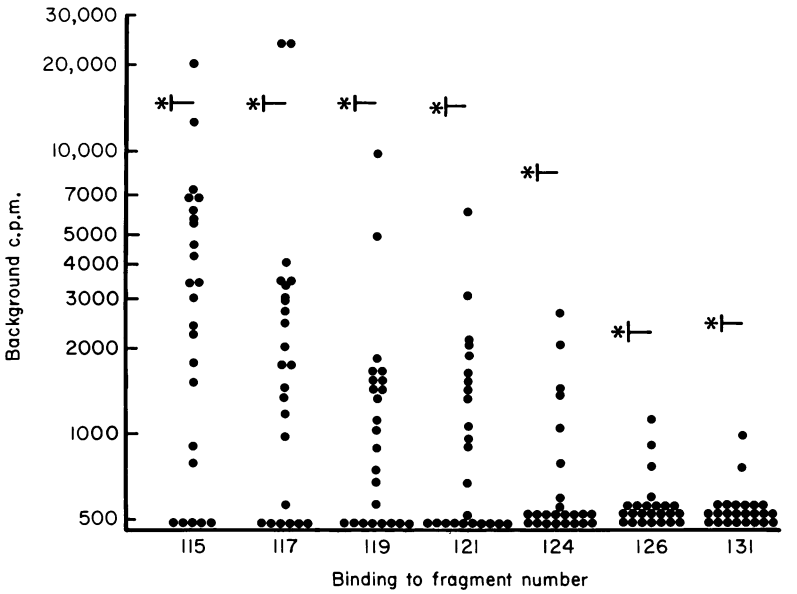

Figure 3. Binding activity of 24 Hashimoto's disease sera at a dilution of 1/50 to thyroglobulin fragments expressed as c.p.m. bound, minus the background level of the sera on unsensitized plates. * = The binding level of a positive control rabbit anti-human Tg (1/100). There was no activity detectable against fragment 134.

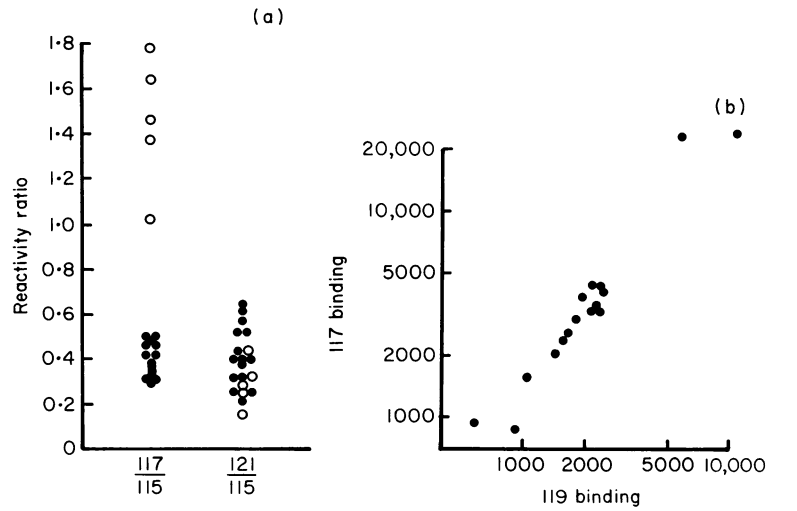

Figure 4. Ratio of reactivity of 20 Hashimoto's disease sera against fragments 117 or 121 compared with 115 (a). The activity against 117 subdivides the patients into two groups (● and ○), indicating that some epitopes on 117 are recognized by only a fraction of the patients. The same patients do not produce a bimodal distribution of reactivity when tested against 121, indicating that the additional epitope(s) is absent from 121. In (b), the comparative binding activity of the 16 highest titred sera is shown against 117 and 119 expressed in specific c.p.m. The high degree of correlation suggests that 117 and 119 express identical epitopes.

121 which are recognized by only a minority of patients. Since 117 is smaller than 115, either this epitope has become exposed during the digestion process, or fragment 117 overlaps with 115 in that they share some, but not all, of the same epitopes. When the same sera were run on different batches of fragments, there was some variation in the numbers of counts bound, but the sera always resolved into the same two groups. Similarly, when sera were taken from patients at different times, their reaction pattern (i.e. the ability to recognize the epitope in 117 and 119 or not) remained unchanged. This implies that some Hashimoto patients react to parts of the molecule which others either do not recognize or do not react to.

Recognition of Tg fragments by T cells

In order to determine whether T cells recognize the same parts of the Tg molecule as B cells, the fragments were used in stimulation assays on Tg-specific T-cell lines. The T-cell lines were originally prepared from spleens of mice immunized with mouse Tg, but were subsequently found to be stimulated equally well with human Tg. The data shown in Fig. 5 demonstrate stimulation of the Tg-specific lines by the fragments. All the fragments except 134 were capable of producing stimulation of the Tg lines, but did not stimulate a PPD-specific line (control for non-specific mitogenic activity of the fragments). It is notable that fragments 121, 124, and 126 produced the most efficient stimulation and that, by contrast with the antibody binding above, fragments 115 and 117 were relatively ineffective. The data in Fig. 5 show the stimulation produced by an optimal concentration of the fragment up to a maximum of 20 μg/ml, but the conclusion that smaller fragments are more effective than larger ones is the same when expressed in equivalent molarity of fragments. It appears then that the autoantibodies recognize epitopes on the larger fragments, while T cells recognize determinants on the smaller ones. Ideally, these studies should be performed with Tg-specific lines from Hashimoto patients, but these are not yet available; it is, therefore, important to exclude the possibility that mouse T and B cells recognize Tg in a

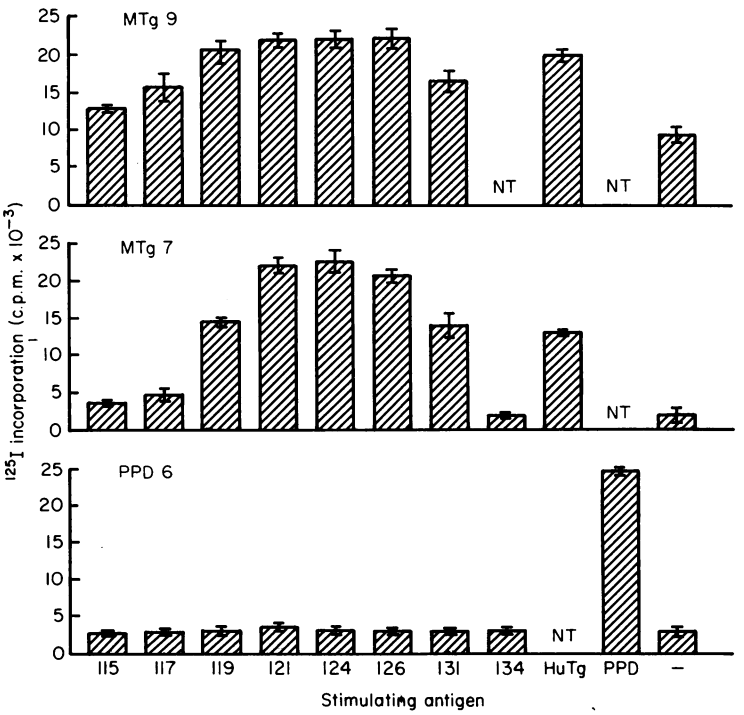

Figure 5. Stimulation of Tg-specific cell lines (MTg 9 and MTg 7) and PPD-specific cell line (PPD 6) by human thyroglobulin fragments (115–134), whole human thyroglobulin (HuTg), PPD and no antigen (–). The results are expressed as c.p.m. (mean ± SEM) of [^{125}I]deoxyuridine incorporated during 16 hr, following 2 days' culture with the antigen. NT = not tested.

different way to human T and B cells. This point is partly answered by determining whether mouse and human B cells recognize the same parts of the Tg molecule as indicated below.

Induction of anti-Tg antibody by fragments

It is possible to immunize mice with fragments to determine the part of the molecule in which antibodies are made. The results are presented in Table 2. This shows that antibodies in the mice are primarily directed to determinants in the larger fragments, and the larger fragments are the most efficient inducers of antibody. This is analagous to the situation seen in the Hashimoto sera, and demonstrates that mouse T cells and B cells are optimally stimulated by different fragments; the implications of this are discussed below.

Table 2. Immunogenicity of human thyroglobulin fragments

Immunizing fragment	Antibody titre to fragment*					
	115	117	119	121	124	126
115	99	344	149	625	17	17
117	1612	3125	1886	3700	625	1111
121	1176	1470	1162	1785	238	869
124	–	17	14	38	–	–
126	–	16	–	24	–	–

* Expressed as the median reciprocal titre of serum giving 5000 c.p.m. in a standard RIA, derived by interpolation from binding curves. Three CBA mice were immunized in each group.

DISCUSSION

Enzyme digestion of thyroglobulin has been attempted previously in order to separate epitopes or particular antigenic domains. Unfortunately, since the Tg molecule is very large, it expresses a large number of potential epitopes which can be recognized by xenogeneic antibodies (Heidelberger, 1938); however, the number of epitopes recognized by autoantibodies is much less and may be as few as 4–6 per molecule in Hashimoto's disease sera (Nye *et al.*, 1980). This means that there is a real hope of separating the epitopes by a suitable method. The fractions we have used are not completely homogeneous on ion exchange chromatography and, although some of the heterogeneity is derived from the intact Tg molecule (Davoli *et al.*, 1982), it might be possible to obtain further fractionation of epitope containing peptides by additional separation procedures. One problem with enzyme digestion and fractionation is that some epitopes may be destroyed. Studies with papain and pepsin digests of thyroglobulin have shown that it is possible to retain antigenicity in quite small fragments (Stylos & Rose, 1969). Nevertheless, it is clear from our results that even V8 protease, which produces large fragments and is highly site-specific, also damages or destroys some of the epitopes recognized by monoclonal antibodies. There also appear to be epitopes present in the intact (19S) molecule which are absent from the largest fragment (115) which has a molecular weight commensurate with a half molecule (19S thyroglobulin is a dimer). It must be assumed that either this epitope is formed from both halves of the molecule, or that it has been destroyed by a single clip or a small excision from the half molecule. In this context, it is worth noting that 8 M urea treatment of human Tg did not affect the ability of the antigen to bind human autoantibodies, although precipitation reactions were abolished (Salabè *et al.*, 1973). We also found that the urea treatment did not affect antibody binding in RIA. As in other experiments (Stylos & Rose, 1969), we have found that the immunogenicity of fragments in mice resembles their antibody-binding capacity.

The observation of the heterogeneous binding of Hashimoto sera antibodies to fragment 117 implies both that this fragment expressed more than one autoantigenic epitope, and that the recognition of these epitopes varies between patients. This heterogeneity may explain why others have had difficulty in determining whether the autoantigen expresses four or six epitopes (two or three per half molecule) (Nye *et al.*, 1980). At present, the numbers involved are too small to analyse the genetics of this difference, but there is no immediately evident correlation with HLA-D type.

The finding that T-cell lines respond optimally to the smaller fragments (124, 126, 131) while the antibodies bind more efficiently to the larger fragments could be explained in one of two ways: either the determinants recognized by the T cells are preferentially expressed in the smaller fragments, or the digestion procedure has in some way contributed to the antigen processing which takes place before presentation to the T cells. The same uncertainty applies to stimulation of T cells by xenogeneic Tg. For example, it has been shown that soluble bovine Tg is a

good inducer of antibody production but is not so effective at priming T cells (Romball & Weigle, 1983), although it is not known whether this depends on the T-cell repertoire or the way the Tg is processed. Since the smaller fragments which stimulate the T cells are subfragments of the larger ones, as detected by monoclonal antibody binding (Table 1), it is assumed that any determinants recognized by the T cells on the small fragments would probably be present on the larger ones, unless they had been generated enzymatically. Possibly digestion of the Tg may have facilitated antigen processing for presentation to T cells which recognize small peptide sequences, while it has destroyed the conformation of epitopes recognized by the B cells. It is clear, however, that the majority of epitopes are missing from the smaller fragments as judged by their reduced ability to bind antibody, whereas the T-cell stimulating epitope(s) is not. In some systems, different T-cell populations are stimulated by different epitopes (Benjamin *et al.*, 1984), but the T-cell lines used here are Lyt 1^+, 2^-, and so we assume that these conclusions apply to helper/inducer T cells.

It is not certain that there is a single cross-reacting antigenic determinant on mouse and human Tg recognized by the T cells of all mouse strains, since the responder status of mice of different haplotypes is not always the same for the two antigens (compare Vladutiu & Rose, 1971 with Aihara *et al.*, 1983). The CBA mice used here are H-2^k high responders for both mouse and human thyroglobulin, and the finding that a line grown out with mouse thyroglobulin also reacts to human thyroglobulin strongly implies that, at least in this strain, there is a cross-reacting determinant on both mouse and human Tg recognized by the T cells.

There has been some debate as to the relative importance of T and B cell autoreactivity in thyroiditis. In experimental autoallergic thyroiditis, the development of lesions is not necessarily related to antibody titres (Romball & Weigle, 1983), and thyroglobulin-specific cell lines are able to induce thyroiditis in recipients (Maron *et al.*, 1983). We believe that by determining which epitopes on thyroglobulin are recognized by B cells and which by T cells, it may be possible to selectively direct therapy at particular populations of lymphocytes, e.g. to kill autoreactive T-helper cells or to stimulate T-suppressor cells. Either antigen or anti-idiotype could be used to selectively stimulate particular autoreactive clones or to target toxic molecules onto them (Roitt *et al.*, 1981; Rennie *et al.*, 1983). In this context, antigen fragments can be used to delineate the epitope specificity of B cells and T cells, and may ultimately be used in selective immunotherapy.

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