Restricted antigenicity of thyroxyls in human thyroglobulin

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Interactions between two human iodothyronine-binding autoantisera and three preparations of human thyroglobulin (Tg) were not proportional to the latter's thyroxyl residue content. Probably only one of the several thyroxyl-containing sites in Tg reacted with the immunoglobulins from both antisera. In the case of one of the antisera, which was thyroxine (T₃)-specific, the thyroxyl residue was the immunodominant feature of the antigenic site. The other antiserum, which had a specificity for 3,5,3'-'tri-iodothyronine (T₄), recognized different determinants around the same thyroxyl residue, but this residue was not itself an important element of the binding site. Thus, despite the specificity for T₄ free in solution, the presence of T₄ in the complete antigenic site was tolerated, since other structures supplied the bulk of the binding energy. 'Specificity' of this antiserum for T₃ free in solution is therefore coincidental and need not be ascribed to the presence of T₄ in the original immunogen. Some results obtained in these studies may be interpreted as supporting the possibility that a modified Tg was the immunogen for the generation of these naturally occurring human antisera.

Thyroglobulin (Tg) is a heterogeneous glycoprotein (mol. wt. 660 000) which is synthesized within the thyroid gland. Some of its tyrosine residues are subsequently iodinated and pairs of these iodothyrosines are enzymically coupled to form the iodothyronines (80–95% T₄, 5–15% T₃, and the balance rT₃), which remain covalently linked within the peptide backbone of the protein. This iodinated Tg is stored within the thyroid follicles. The protein is always heterogeneous with respect to its iodothyronine content, both in type and number, owing to variations in iodine supply and the rate of secretion of thyroid hormone, but may contain up to eight such residues (Marriq et al., 1980). The process of thyroid-hormone secretion involves endocytosis and enzymic breakdown of Tg with liberation of T₄, T₃ and rT₃. During this process some Tg is released into the blood. Thyroglobulin is thus a normal constituent of blood, certainly from the time of birth and probably earlier, but despite this, autoantibodies to Tg commonly develop, often in association with thyroid disease. It is not known if native Tg is the immunogen giving rise to the antibodies in this situation as a consequence of some fault in immune surveillance, or whether the structure of Tg has been modified in some way so as to render it immunogenic.

Some human autoantibodies to Tg are directed against sequences in the molecule which include T₃ and these also bind T₄ free in solution (Pearce et al., 1981). Autoantibodies interacting specifically with T₃ have also been described. It is assumed that these latter are also generated against thyroglobulin, but it is not known whether the original immunogenic site contained tri-iodothyronine as a result of incomplete iodination of tyrosine residues or if an 'imperfect' autoantibody was produced against a T₄-containing sequence.

This study was undertaken firstly to establish whether all of the thyroxyl residues within Tg can interact with antibodies and, if not, to identify differences between thyroxyl-containing sites, and secondly, to confirm that T₃-specific autoantibodies are produced against thyroglobulin and to explain how they may be generated by a predominantly T₄-containing immunogen. To these ends we have examined the antigenic properties of iodothyronine residues within native Tg and enzymically produced fragments of Tg with the aid of three different antisera: two spontaneous human antisera which interact with iodothyronines and one ovine antiserum to T₄. One of the human antisera bound T₄ and the other T₃. The sheep antiserum had been

Abbreviations used: Tg, thyroglobulin; T₄, thyroxine (3,5,3',5'-tetraiodothyronine); T₃, 3,5,3'-tri-iodothyronine; rT₃, reverse T₃ (3,3',5'-tri-iodothyronine); DCC-trypsin, diphenylcarbamoyl-trypsin.
raised by immunization with a T₄-bovine serum albumin conjugate.

Materials and methods

Antisera

(1) hT₄Ab. This was a human serum from a hypothyroid patient containing autoantibodies to thyroglobulin; 70% of these antibodies reacted with T₄-containing sites in the protein and also had a strong specificity for T₄ free in solution. Full characterization of this antiserum has been reported (Pearce et al., 1981). By using [¹²⁵I]T₄ as tracer, the cross-reactivities with the antiserum were (that for T₄ being arbitrarily defined as 1.0): T₃, 0.01; rT₃, 0.004; and Tg, 1.0–2.2, depending on the preparation.

(2) hT₄Ab. This was a human serum found by chance in a euthyroid individual containing antibodies which had a high specificity for T₄ free in solution. This serum has not been described previously. By using [¹²⁵I]T₄ as tracer, cross-reactivities with the antiserum were (T₃ = 1.0): T₄, 0.05; rT₃, 0.0005; and Tg, 0.3–0.8, depending on the preparation.

(3) sT₄Ab. This was a commercial product from Advanced Laboratory Techniques (Tunbridge Wells, Kent, U.K.) raised in sheep against a T₂–bovine serum albumin conjugate. It was shown previously (Pearce et al., 1981) to cross-react with human thyroglobulin through thyroxyl residues.

All three antisera were stripped of endogenous iodothyronines before use by multiple (>8) passes through a column of Amberlite CG 400 ion-exchange resin (1 ml/ml of serum). Pooled normal human serum used as an additive in binding studies was also stripped in this way.

Preparation of human thyroglobulin

Three samples of thyroid tissue were obtained 24–36 h post mortem from one hyperthyroid (TgC) and two euthyroid (TgR and TgE) individuals and frozen at −30°C until use. The tissue was thawed, finely chopped and extracted for 2 h with phosphate buffer (0.05 mol/litre), pH 7.4, containing 6-amino-hexanoic acid (0.05 mol/litre) (3 ml/g of tissue). The extract was centrifuged at 300 g for 15 min and the clear red supernatant chromatographed in 5 ml portions on a column (2.5 cm x 38 cm) of Sepharose-6B. The Tg peak was located by a radioimmunoassay for thyroxine, which also detects Tg, and coincided with a u.v. (280 nm)-absorbing peak. Those fractions within the peak with protein concentrations >70% of that found at the centre were pooled, concentrated by pressure dialysis and stored frozen at −30°C until required. The thyroglobulin yields obtained were: TgC, 17.2 mg from 1.36 g of tissue; TgR, 167.8 mg from 5.57 g of tissue; and TgE, 41.4 mg from 1.72 g of tissue. On 5% (w/v)-polyacrylamide-disc-gel electrophoresis at pH 8.6 (Ornstein, 1965; Davis, 1965), each Tg preparation showed two closely spaced bands staining approximately equally with Amido Schwarz, with no evidence for faster- or slower-running components.

Iodothyronine contents of thyroglobulins were determined by conventional radioimmunoassays of total enzymic digests of the proteins.

Enzymic degradation of human thyroglobulin

DCC-trypsin was obtained from Seravac Laboratories, Maidenhead, Berks., U.K.; chymotrypsin, Pronase P and aminopeptidase M were from Sigma (London) Chemical Co., Poole, Dorset, U.K.

All steps were carried out in glass containers to avoid the irreversible losses that occur on plastic surfaces. Tg (4–20 mg/ml) was reduced in 8 M-urea by using 2.43 µl of mercaptoacetic acid/mg of Tg at pH 10.5 for 90 min at room temperature, as recommended by Pitt-Rivers (1976). The pH was then lowered to 8.6 with HCl and the reduced Tg alkylated with 2.23 µg of iodoacetic acid/mg of Tg for 2 h at room temperature. [Free iodine in the iodoacetic acid was removed before use by extraction with light petroleum (b.p. 60–80°C) in order to prevent iodination of tyrosine or thyronine side chains.] The reduced and alkylated Tg was dialysed against 0.1 M-NH₂HCO₃ and quantitatively transferred to a glass container. All subsequent steps were carried out in the same container to avoid manipulative losses and thus to allow calculations of molar quantities of peptides by reference to the original Tg solution. After each step, a known fraction of the sample was removed for study. The reduced and alkylated Tg was digested in turn with: DCC-trypsin (30 µg/mg of Tg) for 48 h at 37°C; chymotrypsin plus Pronase P (both 44 µg/mg of Tg) for 55 h at 37°C; and finally, aminopeptidase M (16 µg/mg of Tg) for 24 h at 37°C. The need for a lengthy tryptic digestion at a high enzyme/substrate ratio was established by fractionating representative Tg tryptic digests on a column of Sephadex G-25 in 0.05 M-pyridine/acetate, pH 6.0, after 24 or 48 h incubation at 37°C. After 24 h incubation two peaks of immunoreactivity were detected by a T₄ radioimmunooassay: one close to the void volume of the column the other at a volume corresponding to a mol.wt. of about 1500. With further digestion all the immunoreactivity was found in the lower-molecular-weight region. Analysis of the digest after chymotrypsin-plus-Pronase P treatment by ion-exchange chromatography in a conventional amino acid analyser (Beckman 121MB) revealed a few amino acids and many short peptides. The subsequent aminopeptidase treatment degraded the material to free iodothyronines and amino acids. Immunoreactive peptides were not isolated at any stage of digestion in order to avoid the losses...
that would have prevented a quantitative assessment of changes in cross-reactivity as Tg was fragmented. It was also not necessary, since study could be restricted to T4-containing fragments by using 125I-labelled iodothyronine tracers and the specificity of the antisera.

Antibody-binding studies

Pure iodothyronines for standards were purchased from Henning, Berlin, Germany. High-specific-radioactivity (>1200 μCi/μg) [125I]T4 and [123I]T3 were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Binding experiments were carried out in barbitone buffer (0.1 mol/litre), pH 8.6, containing 8-anilino-naphthalenesulphonic acid ammonium salt (955 mg/litre) and bovine serum albumin (2 g/litre). Each test tube contained the following components dissolved in the buffer to a total volume of 550 μl: the amount of test substance, tracer [125I]T4 or [123I]T3, an amount of antiserum that would bind about 50% of the tracer and 50 μl of iodothyronine-free pooled normal human serum. The mixtures were incubated for 20h at room temperature, after which antibody-bound [125I]T4 was precipitated by the addition of 1 ml of 30% (w/v) poly(ethylene glycol) 6000. After centrifugation the supernatant was removed by suction and the radioactivity in the pellet determined.

Presentation of results

Dose–response curves for the interaction of substances with the antisera are presented as log–linear plots of the molar quantity of substance against the percentage of 125I-labelled tracer bound to antibody, with duplicate determinations at each point.

Molar quantities of Tg were based on protein estimation (Lowry et al., 1951) assuming a mol.wt. of 660000. Values for the products of enzymic digestion of the protein were calculated from the molar quantity of Tg from which they were derived, without correction for the presence of the subunits.

Interactions of substances with the antisera were assessed both in terms of cross-reactivity, defined as the ratio of the molar quantities of two substances required to achieve a given degree of inhibition of tracer binding to antibodies (usually 50% of the maximum), and also in some cases the shape of the dose–response curve of tracer binding in the presence of increasing quantities of test substance.

Results

Interactions with the thyroxine-specific antisera hT4Ab and sT4Ab

Human thyroglobulin preparations TgR and TgE from euthyroid tissue contained similar amounts of iodothyronine residues per mol but more than did TgC extracted from thyrotoxic tissue (Table 1). All three Tg preparations inhibited the binding of [125I]T4 tracer to the antisera hT4Ab and sT4Ab in a dose-dependent fashion (Fig. 1). The dose–response curves were parallel with those for T4, and full inhibition of binding could be achieved, confirming our previous observations (Pearce et al., 1981). However the cross-reactivities of TgR, TgE and TgC versus T4 with both antisera bear no relationship to either the thyroxyl or total iodothyronine contents of the proteins (Table 1 and Fig. 1).

Individual iodothyronine residues within Tg must therefore vary in their accessibility and reactivity with the immunoglobulins of these sera. Despite TgR and TgE containing 4.2 and 4.0 thyroxyl residues respectively per mol, they had cross-reactivities with hT4Ab close to that of T4 (i.e. 1.1 and 1.0) (see Table 1 and Figs. 1c and 1e). On the other hand, TgC, with only 1.1 residues of thyroxine per mol, cross-reacted 1.5 times better than did T4 (Table 1 and Fig. 1a). It is therefore likely that preparations TgR and TgE contain thyroxyl residues not available to bind with hT4Ab and, in view of the high reactivity of TgC, only one iodothyronine-containing site may be capable of interaction with this antisera. Steric hindrance to the approach of a second immunoglobulin after the first has bound is possible and will be discussed below.

The dose–response curves for the inhibition of tracer binding by enzymic digests are plotted according to the molar quantities of Tg from

<table>
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<th>Thyroglobulin preparation</th>
<th>Content (residues/mol of Tg)</th>
<th>Cross-reactivity (Tg against T4)</th>
<th>Cross reactivity (Tg against T3)</th>
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<tr>
<td>TgR</td>
<td>4.2 0.2 0.1</td>
<td>Antiserum hT4Ab 1.1 0.7 0.3</td>
<td>Antiserum hT3Ab n.d. 0.8</td>
</tr>
<tr>
<td>TgE</td>
<td>4.0 0.2 0.1</td>
<td>1.0 0.4</td>
<td></td>
</tr>
<tr>
<td>TgC</td>
<td>1.1 0.1 0.1</td>
<td>1.5 0.4</td>
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Table 1. Iodothyronine content of the human thyroglobulin preparations and their cross-reactivities against T4 for binding to the T4-specific antisera hT4Ab and sT4Ab and against T3 for binding to the T3-specific antisera hT3Ab

Abbreviation used: n.d., not determined.
Fig. 1. Studies with the T₄-binding human and sheep antibodies
Inhibition of ¹²⁵I-labelled thyroxine tracer binding to the human anti-T₄ autoantibody, hT₄Ab (a,c,e) and to the sheep anti-T₄ antibody, sT₄Ab, (b,d,f) by the three thyroglobulins (■), their tryptic digests (▲), their total digests (●) and thyroxine (○). Molar quantities of the enzyme digests were determined from the quantities of thyroglobulin from which they were derived.

which they were derived without correction for the presence of subunits or for thyroxyl or total iodothyronine content. Enzymic degradation generally increased the cross-reactivity of each Tg preparation, resulting in dose–response curves displaced to the left of the respective Tg curve (Table 2 and Fig. 1).

In most cases the reactivity increased in the order Tg < tryptic digest < total enzymic digest, presumably because increasing degradation of the molecule increased accessibility of thyroxyl residues. The exception was the lesser interaction of the tryptic digest of TgC with the human autoantibody hT₄Ab than that of intact TgC (Table 2 and Fig. 1a); the reactivity of the total digest of TgC, however, was greater than either of these.

Increased reactivities in the digests reflect either the exposure of thyroxyls previously buried within the protein, or the removal of steric constraints that had prevented immunoglobulins from simultaneously binding to all exposed T₄ residues. A third variable is evident in the results from preparation TgC. It contained a mean of 1.1 mol of T₄ residues/mol and thus would have generated only 1.1 mol of T₄-containing fragments after tryptic digestion. This
Table 2. Relative effectiveness of substances at inhibiting tracer binding to the three antisera, hT₄Ab, sT₄Ab and hT₃Ab

<table>
<thead>
<tr>
<th>Antiserum</th>
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<tr>
<td></td>
<td>hT₄Ab</td>
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<td>TgC</td>
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<tr>
<td>Tryptic digest</td>
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<td>Total digest</td>
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<tr>
<td>T₄</td>
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<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Amount (pmol)</th>
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<tr>
<td></td>
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<tr>
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<tr>
<td>T₄</td>
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<td>T₃</td>
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<table>
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<tr>
<th>Antiserum</th>
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<tr>
<td></td>
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<tr>
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<tr>
<td>T₄</td>
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† Quantities for the enzymic digests were derived from the quantity of parent Tg from which they came without correction for the number of reactive fragments produced.

The Table shows the amounts of the human thyroglobulin preparations, their enzymic digests†, and T₄ and T₃ required to achieve 50% inhibition of tracer binding to the three antisera. Data taken from the experiments illustrated in Figs. 1 and 2. Values marked with an asterisk were obtained from curves not parallel with others in the same experiment. Cross-reactivities derived from these values would therefore not be valid at other levels of inhibition of tracer binding. Nonetheless these curves do lie wholly to the left (TgR and TgE, Fig. 1c and 1e) or wholly to the right (TgC, Fig. 1a) of those of their parent Tg, so that the general statements of increased or decreased cross-reactivities made in the text for these digestes are valid.

Cross-reactivities of all three Tg preparations versus T₄ for binding to the sheep antiserum were lower than with the human antiserum (Table 1). The dose–response curves were set to the right of that for T₄ and parallel with it (Figs. 1b, 1d and 1f). This is not unexpected, since the sheep antiserum was raised against T₄ linked to an albumin side chain rather than within the peptide backbone as in Tg, and it would thus recognize only the thyroxyl moiety. This supports the inferences above that structural features in Tg other than the T₄ moiety are involved in binding to hT₄Ab.

As before, the cross-reactivities of the three Tg preparations with sT₄Ab are not proportional to either their thyroxyl or total iodothyronine contents. Both TgC and TgE, with thyroxyl residue contents of 1.1 and 4.0 respectively per mol, had the same cross-reactivity (0.4) versus T₄ (Table 1 and Figs. 1b and 1f) in binding to sT₄Ab. TgE therefore contains thyroxyl residues which are inaccessible to the sheep immunoglobulins and, as was inferred for the human serum, probably only one thyroxyl was involved in the interactions, since TgC has only one. This must be the same one that reacts with the human serum hT₄Ab. However, TgR is 1.8 times as effective as TgE at inhibiting [¹²⁵I]T₄ binding to sT₄Ab (Tables 1 and 2 and Figs. 1d and 1f), although both contain similar average numbers of thyroxyl residues per mol (4.2 and 4.0 respectively). This can only be explained in terms of differences in reactivity between the thyroxyl residues, although the precise reason is not clear. Most probably TgR contains an additional thyroxyl accessible to the sheep but not to the human antibodies, so that steric hindrance to the approach of more than one immunoglobulin does not explain all the differences in reactivity. It would seem, therefore, that thyroxyl synthesis in vivo is possible at more than four sites. Each of the three thyroglobulin preparations studied here contains a thyroxyl at the site with which human serum hT₄Ab interacts. But the additional thyroxyl residues in TgR and TgE would seem to be differently distributed among more than three sites.

Dose–response curves for the inhibition of [¹²⁵I]T₄ binding to the sheep antiserum by the tryptic digestes are parallel with those of Tg and T₄ (Figs. 1b, 1d and 1f), in contrast with our experience with the human serum. This confirms that the sheep immunoglobulins recognize only the thyroxyl moiety and are insensitive to conformational determinants and the presence or absence of neighbouring amino acid side chains of Tg.

To summarize the results thus far. It is clear that the reactivities of thyroxyls in Tg towards both antisera vary. Possibly only one thyroxyl can react with the human autoantibody and this one is in a site
that requires conformational determinants and/or neighbouring amino acids present only in the native thyroglobulin for full reactivity. The thyroxyl which reacts with the human antibody probably reacts with the sheep antibody, but TgR seems also to contain another thyroxyl that interacts only with sT4Ab.

**Interactions with T3-specific human serum hT3Ab**

Only TgR, TgC and their degradation products were tested against this antiserum. Both Tg preparations inhibited the binding of [125I]T3 tracer to the antiserum in a dose-dependent fashion, and full inhibition could be obtained (Fig. 2), thus indicating that the antibodies were indeed raised against human Tg.

Both thyroglobulin preparations cross-reacted with T3 better than would be expected from their low T3 contents (Table 1 and Fig. 2), and once again TgC was the more potent of the two, showing that reactivity with this antiserum is also not related to the iodothyronine content of thyroglobulin. Since the 4.2 mol of thyroxyl residues of TgR do not confer more reactivity than the 1.1 mol of such residues in TgC, it is likely that the hT3Ab reacts with one site only in Tg. If both hT3Ab and hT4Ab react with one site only, and that is the one present in TgC, then it must be concluded that they interact with the same epitope. Both Tg preparations were much more reactive than was T4 (Table 2 and Fig. 2).

Changes in cross-reactivity on enzymic degra-
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(Fig. 2), in contrast with the experience with the T₄-specific human serum (Fig. 1a, 1c and 1e). This may indicate that the antibody population was reasonably homogeneous.

Discussion

Direct analytical methods applied to Tg preparations from several species have shown that, in general, the first tyrosine residues to be iodinated are also the first to couple into iodothyronines (Lamas et al., 1974; Dème et al., 1976; Gavaret et al., 1977; Vignal et al., 1978) and that the different thyroxsyls in Tg are synthesized in an order that is controlled by the tertiary structure of the protein (Rolland et al., 1972; Lamas et al., 1974; Dème et al., 1976; Gavaret et al., 1977; Maurizis et al., 1981). It is implicit in these observations that the iodothyronine-synthesizing sites in Tg differ in some way. Our studies on the antigenicity of these sites agree with this conclusion. Preparation TgC was the most reactive with both human antisera, although it contained only a mean of 1.1 mol of T₄ residues per mol, which must largely represent the first T₄ residue to be synthesized. The human antiserum Tg₁Ab and hT₄₁Ab are therefore directed against this primary site of T₄ synthesis. Preparations TgR and TgE contained an average of 4.2 and 4.0 mol of thyroxyls per mol, but did not have cross-reactivities with hT₄₁Ab and hT₄₂Ab greater than that of TgC (Table 1). Since they must of necessity contain the first T₄ residue to be synthesized, we suggest that this residue alone is responsible for their reactivity with human autoantisera. This suggestion has important consequences in understanding the development of iodothyronine-binding autoantisera to human thyroglobulin, since it implies that immunogenicity may be a feature not of iodothyronine residues generally but of the thyroglobulin structures within which they are set.

Why is this site immunogenic? There are two possible explanations: a failure of immune surveillance or an acquired modification to this part of the molecule rendering it immunogenic. In favour of the second possibility is the difference in properties of the tryptic fragments and native Tg with respect to their interactions with the T₄-specific antiserum (hT₄₁Ab) (Figs. 1a, 1c and 1e). If the immunogen did indeed have a structure different from native Tg, then flexible tryptic fragments might more readily conform to the antibody-binding site than would the antigenic site when constrained within the whole native protein. Differing degrees of conformity by tryptic fragments and native Tg with each of the different members of the antibody population would then result in the different shapes of dose-response curves seen in Figs. 1(a), 1(c) and 1(e). In support of this we have found (A. Bond & P. G. H. Byfield, unpublished work) that reduced and alkylated Tg with ruptured disulphide bonds also gives dose-response curves different from native Tg, but having the same shape as those given by tryptic digests when inhibiting the binding of [¹²³I]T₄ to hT₄₁Ab. Additional support is also found in the experiments of Ochi et al. (1972), who showed in rabbits that denatured human Tg produced iodothyronine-binding antibodies, but that native Tg did not. It remains possible, however, that the antigenic site straddles two adjacent peptide chains which are parted by tryptic digestion and that the loss of a determinant is responsible for the effects.

Conclusions

The present study of the interactions of human thyroglobulin with two human autoantisera and one ovine antiserum to a T₄-albumin conjugate has thrown light on the antigenic properties of thyroxyl-containing sites in Tg. (i) It has confirmed that a T₄-specific antiserum was raised against human Tg and given an explanation for its production by a presumed T₄-containing immunogen. (ii) The extent of the interaction between each thyroglobulin and each antiserum was unrelated to the number of iodothyronine residues within each thyroglobulin molecule. Indeed, as far as the two human antisera were concerned, TgC, with the least iodothyronine content, was the most effective preparation at inhibiting [¹²³I]iodothyronine tracer binding to antibodies. (iii) The human T₄-specific antiserum could react with only one of the possible T₄-containing sites in Tg; the thyroxyl moiety was an immunodominant feature of the antigenic site and the interaction also involved adjacent structures in native Tg. (iv) It was inferred that the human T₄-specific antiserum also reacted with this same site, albeit with a different specificity; there was a high degree of interaction with neighbouring amino acids and the iodothyronine was only a minor determinant. The presence of T₄ in the antigenic site in Tg could therefore be tolerated and the production of a coincidentally T₃-specific immunoglobulin was an adequate immune response. (v) The antigenic site for these two antisera is around the first iodothyronine residue synthesized in Tg.

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References


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