Structural basis for the reaction of 3,5,3'-tri-iodothyronine-specific antibodies with thyroxine-containing thyroglobulin

Peter G. H. BYFIELD, Dorothea CLINGAN and Richard L. HIMSWORTH
Endocrinology Research Group, Clinical Research Centre, Harrow, Middx. HA1 3UJ, U.K.

(Received 7 November 1984/4 February 1985; accepted 11 February 1985)

A series of human autoantibodies against thyroglobulin (Tg) which exhibit different specificities for iodothyronines were studied. The ability of a thyroxine (T_4)-containing peptide (T_4P) isolated from human thyroglobulin (Tg) to displace [^{125}I] T_3 from human T_3 -specific autoantisera was 11–50 times greater than that of T_4 alone. These antisera therefore strongly recognize amino acids adjacent to T_4 in the Tg structure. This was confirmed when a Tg preparation (Tg[0.05]) containing an average of only 0.05 of a T_4 residue/molecule and much less T_3 had good cross-reactivities with these antisera. Cross-reactivities of other Tg preparations with different T_4 contents increased only slowly with increase of T_4 content up to a mean of 6.6 residues/molecule and were not proportional to T_3 content. In contrast, cross-reactivities with a human T_4 -specific autoantiserum were strongly dependent on T_4 content. Tg[0.05] was 500 times less reactive than T_4P and 615 times less than T_4 . Cross-reactivities rose rapidly as the T_4 content of Tg preparations increased from a mean of 0.05 to approx. 1–2 residues/molecule. Thyroxine is therefore a dominant feature of the antigenic site for this antiserum. There was little further increase in cross-reactivities for those Tg preparations containing up to an average of 6.6 residues T_4 per molecule, confirming previous conclusions that all T_4 -containing sites are not immunologically identical and that autoantibodies exhibit a preference for particular sites on Tg. Similar conclusions were reached for a non-specific iodothyronine-binding antiserum. These results indicate that iodothyronine specificity in human autoantisera is not necessarily determined by the iodothyronine present in the immunogenic area, but by the precise site selected by the immune response. T_4 - or non-specific antibodies have thyroxine as a dominant feature of the antigenic site. T_3 -specific antibodies have the thyroxine residue as a peripheral feature of the binding site, and it is not necessary to postulate that T_3 was part of the immunogen or is required in the epitope. These antisera may have value in mapping the hormonogenic regions in Tg from human and other species.

Autoantibodies to Tg are often found in association with thyroid disease and occasionally in healthy adults. In some individuals the autoantibodies may be generated against a hormonogenic region on the protein where T_4 is synthesized

Abbreviations used: Tg, thyroglobulin; T_4 , thyroxine (3,5,3',5'-tetraiodothyronine); T_3 , 3,5,3'-tri-iodothyronine; Tos-Phe-CH₂Cl-trypsin, tosylphenylalanylchloromethane ('TPCK')-treated trypsin; T_4P , T_4 -containing peptide; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; Tg[0.05], a thyroglobulin preparation containing an average of only 0.05 residue of thyroxine per molecule (similarly for Tg[0.3] etc.).

(Pearce *et al.*, 1981). These antibodies also cross-reacted with free iodothyronines. As would be expected, their specificity varies; some are quite specific for T_4 and some will bind any iodothyronine, but, interestingly, some are quite specific for T_3 . It might be suggested that the particular immunogenic molecule which provoked the immune response may have been fortuitously underiodinated and have contained T_3 in place of T_4 . However, in our previous studies (Byfield *et al.*, 1982; Himsworth *et al.*, 1983) we concluded from preliminary evidence that this was not a necessary postulate and that the immunogen could have contained T_4 , since the immunodominant features of

the antigenic site for a T_3 -specific antibody appeared to be adjacent to the iodothyronine residue. Thus an inadequate reaction with a T_4 residue in the epitope could be tolerated, since the bulk of the binding energy was generated by adjacent features. Specificity for T_3 in solution was therefore coincidental. A necessary corollary is that thyroglobulin molecules without T_4 in this site would react well with the T_3 -binding antisera, but should not bind well to T_4 -specific autoantibodies. To examine these possibilities we collected a series of human Tg preparations with different T_4 contents and also isolated a T_4 -containing heptapeptide fragment of human Tg. These substances, and T_4 and T_3 , were compared for their cross-reactivity with several human autoantibodies possessing different specificities for T_4 and T_3 .

Methods

Preparation of human thyroglobulin

Human thyroid tissue was obtained 36 h *post mortem* or at operation for the removal of toxic multinodular goitre and was frozen at -30°C until use. The tissue was thawed, finely chopped and extracted for 2 h with 0.5 M-sodium phosphate buffer, pH 7.4, containing 0.05 M-6-aminohexanoic acid (3 ml/g of tissue). The extract was centrifuged at 300g for 15 min and the clear red supernatant chromatographed in 5 ml portions on a column (1.5 cm $\times$ 75 cm) of Sepharose 6B. The Tg peak was located by a radioimmunoassay for T_4 , which cross-reacts with Tg, or by protein-concentration measurements (Lowry *et al.*, 1951) and, in some runs, also by u.v. absorption. Central regions of the peak were pooled, concentrated by pressure dialysis and stored at -30°C until required.

Iodothyronine contents of thyroglobulin preparations were determined by conventional radioimmunoassays of total enzymic digests of the proteins. Total digestion of the thyroglobulin samples was carried out at 37°C in 0.05 M-sodium phosphate buffer, pH 7.4, by using, first, an equal weight of Pronase for 24 h, followed by aminopeptidase M (1 mg/50 mg of Tg) for 24 h. Both enzymes were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

Isolation of a T_4 -containing peptide from human Tg

A Tg sample with a low T_4 content (Tg[1.0] of Table 2 below) was selected for this preparation in the expectation that it would contain fewer thyroxine-containing sequences and thus simplify the task. Nonetheless, preliminary studies showed that a lengthy digestion with Tos-Phe- CH_2Cl -trypsin was necessary in order to produce a simple pattern of one T_4 -containing peptide ($T_4\text{P}$) plus

thyroxine in the digest when examined by gel filtration on Bio-Gel P-6 (200–400 mesh). Tg[1.0] (2.53 g) was dissolved in 0.2 M- NH_4HCO_3 (320 ml) and incubated at 37°C with 84 mg of Tos-Phe- CH_2Cl -trypsin (Sigma Chemical Co., Poole, Dorset, U.K.) for 72 h. A further 84 mg of Tos-Phe- CH_2Cl -trypsin was then added and the digestion continued for 72 h. To take advantage of the interaction of iodothyronines with the Sephadex matrix (Lissitzky *et al.*, 1962) to retard selectively T_4 -containing peptides, the digest was chromatographed in 100 ml portions on a column (1 cm $\times$ 28 cm) of Sephadex G-25 (fine grade) and eluted with 0.1 M- NH_4HCO_3 at 18 ml/h, collecting 20 min fractions. After 40 fractions had been collected, the solvent was changed to 0.05 M- Na_3PO_4 to elute thyroxine (Kjeld *et al.*, 1975). Fractions containing this solvent (no. 44 onwards) were neutralized by the addition of 0.75 ml of 0.5 M- NaH_2PO_4 . T_4 -containing compounds were located by a T_4 radioimmunoassay on 0.4 μl samples from each fraction. A single u.v.-absorbing peak extended over fractions 2–25, whereas the main T_4 -containing peak was located in fractions 12–40, showing that retardation had indeed occurred. Fractions 26–40 were pooled and freeze-dried. The contents of fractions 12–25 were pooled (90 ml) and re-chromatographed in the same way in two 45 ml portions. Once again, the activity peak (fractions 6–25) was partly overlaid by the protein peak (fractions 2–14). Fractions 15–25 were therefore pooled with the active pool from the first run.

The combined active pools were then freeze-dried and redissolved in 1.0 ml of 0.1 M- NH_4HCO_3 . This was applied in two 0.5 ml portions to a column (1 cm $\times$ 28 cm) of Bio-Gel P-6, 200–400 mesh, and eluted with 0.1 M- NH_4HCO_3 at 8 ml/h, fractions (0.8 ml each) being collected. Radioimmunoassay showed a peak of activity in fractions 16–18, indicating that no adsorption occurred in this polyacrylamide gel. Chromatographic analysis (cellulose layers; solvent: butanol/pyridine/acetic acid/water, 21:12:2:15, by vol.) showed a single peptide component in these fractions, R_F 0.55, which stained weakly with ninhydrin and strongly with a modified Reindel-Hoppe reagent (Von Arx & Neher, 1963). Amounts of the peptide were determined by amino acid analysis after acid hydrolysis (6 M-HCl, 24 h, 110°C). The yield was 1.12 μmol from 2.53 g (3.83 μmol) of thyroglobulin.

The peptide was refractory to digestion by chymotrypsin, pepsin, papain, thermolysin, leucine aminopeptidase and *Staphylococcus* V8 proteinase. It could be partly degraded by aminopeptidase M, and completely by large concentrations of Pronase. Its amino acid composition was $\text{Asx}_2\text{Thr}_1\text{Ser}_1\text{Glx}_1\text{Ala}_1\text{thyroxine}_1$. The *N*-

terminal residue was aspartic acid/asparagine by dansylation (Hartley, 1970).

Human autoantisera

1. *EB*. Detailed studies of this autoantibody, which is mainly directed against a thyroxine-containing epitope in human thyroglobulin, have been published (Pearce *et al.*, 1981; Byfield & Bond, 1982; Byfield *et al.*, 1982). The antibody cross-reacts with iodothyronines free in solution, but shows a high specificity for T_4 .

2. *SC*. A human serum, found by chance in a euthyroid individual, containing antibodies which had high specificity for T_3 free in solution. Some of its properties have been described (Byfield *et al.*, 1982).

3. *ET, GP and MM*. These sera were referred to us for detailed studies, owing to their observed interference in radioimmunoassays used for the diagnosis of thyroid disease. Their properties have not been described. They exhibit specificity for T_3 .

4. *CK*. This serum was also referred owing to interference with radioimmunoassays for T_4 and T_3 . It did not distinguish between these two iodothyronines.

All antisera were stripped of endogenous iodothyronines before use by multiple (more than eight) 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.

Cross-reactivity studies

Pure iodothyronines for standards were purchased from Henning, Berlin, Germany. High-specific-radioactivity ($>1200 \mu\text{Ci}/\mu\text{g}$) radio-labelled [^{125}I] T_4 and [^{125}I] T_3 were purchased from Amersham International, Amersham, Bucks., U.K. Binding experiments were carried out in 0.1 M-barbitone buffer, pH 8.6, containing bovine serum albumin (2 g/l) and 8-anilidonaphthalene-sulphonic acid ammonium salt (955 mg/l) to prevent tracer binding to serum proteins other than antibodies. Each test tube contained the following components dissolved in the buffer to a total volume of 500 μl : the amount of test substance, tracer [^{125}I] T_4 or [^{125}I] T_3 , an amount of antiserum that would bind 30–50% of the tracer and 50 μl of iodothyronine-free pooled normal human serum. The mixtures were incubated for 20 h at room temperature, after which antibody-bound [^{125}I] T_4 or [^{125}I] T_3 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.

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 ^{125}I -labelled tracer bound to antibody, with duplicate determinations at each point. Molar quantities of Tg were based on protein determination (Lowry *et al.*, 1951), an M_r value of 660 000 being assumed.

Interactions of substances with the antisera were assessed 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. All values were normalized against the cross-reactivity of the T_4 -containing peptide.

Results and discussion

Autoantisera are not generally monoclonal and, in the case of autoantisera to Tg, it is known that individual immunoglobulins in the mixture may be directed against different sites on the Tg molecule (Nye *et al.*, 1980). This is certainly the case for serum EB used here (Pearce *et al.*, 1981), although not for serum SC (P. G. H. Byfield & S. Copping, unpublished work). The use of radiolabelled iodothyronines as tracers, however, allowed studies of these antisera to be restricted to those antibodies directed against iodothyronine-containing sites on Tg.

Six Tg preparations were used in the present study. They were chosen to cover a wide range of T_4 content, extending above and below the average of three to four residues/molecule commonly found. Their T_4 and T_3 contents are listed in Table 1. In general the T_3 contents were about one-tenth those of T_4 ; Tg[0.3] however, contained twice as much T_3 as T_4 but, as will be seen, its binding properties seemed to relate to its T_4 content. These Tg preparations were tested for their ability to interact with six human autoantisera of differing specificities for iodothyronines. Inhibition of the binding of [^{125}I] T_4 or [^{125}I] T_3 , as appropriate, to antibodies was dose-dependent. At sufficiently high concentrations of Tg, T_4 or T_3 , amounts of bound tracer could be decreased to the level of non-specific binding in the system.

Typical examples of dose-response curves for two of the autoantisera studied are shown in Fig. 1. Curves for the percentage of [^{125}I]iodothyronine tracer bound to antibody in the presence of increasing concentrations of Tg were parallel with those given by T_4 and T_3 for all antisera. So too were those given by the T_4 -containing fragment of Tg (result not shown). In a previous study (Byfield *et al.*, 1982), unfractionated tryptic digests of Tg were found to give displacement curves non-parallel with those of T_4 and Tg when tested against the T_4 -specific antiserum EB. The composition of this peptide is consistent with its being the human equivalent of a major T_4 -containing sequence found in

Table 1 *Interactions of human iodothyronine-binding autoantibodies with a series of ligands*

Cross-reactivities relative to the T₄-containing peptide (T₄P, ≡ 1.00) for the displacement of ¹²⁵I-labelled iodothyronine from a series of human antisera by T₄, T₃, T₄P and by a series of thyroglobulin preparations containing different average amounts of T₄ residues.

Ligand	Iodothyronine content (mol/mol)		Antiserum ... Tracer ...	Cross-reactivity						
	T ₄	T ₃		EB T ₄	ET T ₃	MM T ₃	GP T ₃	SC T ₃	CK T ₃	CK T ₄
T ₄	1	0		1.23	0.02	0.09	0.03	0.04	1.34	1.28
T ₄ P	1	0		1.00	1.00	1.00	1.00	1.00	1.00	1.00
T ₃	0	1		0.02	33.81	4.99	17.01	0.91	8.93	2.93
Tg[0.05]	0.05	0.007		0.002	0.40	1.70	0.15	0.01	<0.02	<0.005
Tg[0.3]	0.3	0.57		0.09	1.25	1.23	0.82	0.02	0.30	0.09
Tg[1.0]	1.0	0.17		0.58	—	2.23	1.55	0.07	0.51	0.38
Tg[1.9]	1.9	0.16		1.38	1.69	3.33	1.87	0.31	2.89	0.78
Tg[4.2]	4.2	0.20		1.38	5.16	5.02	3.05	0.25	5.75	1.56
Tg[6.6]	6.6	0.74		3.28	5.25	12.47	7.15	0.58	8.16	2.76
Ratio of cross-reactivities of Tg[6.6] and Tg[0.05] ...				1640	13.1	7.3	48	58	>408	>552

porcine Tg by Marriq *et al.* (1982) and having a core structure of Asp-Asp-T₄-Ala-Thr. There is however, no direct evidence to identify peptide T₄P with an immunogenic site.

Reactions with the T₄-specific antiserum

Cross-reactivity with antiserum EB was strongly dependent on the T₄ contents of the Tg preparations (Table 1). Preparation Tg[0.05], containing an average of only 0.05 of a T₄ residue per molecule of Tg, had a cross-reactivity with this antiserum 615 times less than that of T₄. Cross-reaction of the Tg preparations with this antiserum rose rapidly as their T₄ content increased to equal that of T₄ at between one and two residues per molecule. This residue is therefore a dominant feature of the antigenic site. Reactivity of Tg preparations with higher T₄ contents was only a little higher even at T₄ contents of an average of 6.6 residues/molecule. Similar observations led us to propose previously (Byfield *et al.*, 1982) that this antiserum exhibits a preference for one particular T₄ residue, although at least two are available for binding (Byfield *et al.*, 1984). There is therefore an influence from the local topography on the choice of antigenic site, but the bulk of the antigen-antibody binding energy derives from interactions at the T₄ residue within that site. T₄P, the peptide isolated from Tg, was a little less reactive than T₄ with this antiserum, so the surrounding structures in this peptide sequence provide no additional interactions with the antibodies.

Reactions with T₃-specific antisera

The cross-reactivity of T₄ with the T₃-specific

antisera was, of course, low or very low (Table 1). However, the T₄-containing peptide, T₄P, had a cross-reactivity much higher (11–50 times) with each of these antisera than that of T₄ in isolation. Thus having this amino acid within a natural peptide sequence increases binding to these antibodies. The neighbouring amino acids therefore contribute greatly to the antigen-antibody interactions. A necessary corollary to this finding is that Tg deficient in iodothyronines should react strongly with these antisera. This was confirmed in the results for the cross-reactivity of Tg[0.05] with three of the T₃-specific antisera (ET, MM and GP; Table 1). This preparation of the protein contains minute concentrations only of thyroxine residues, but nonetheless had a cross-reactivity 5–20 times greater than T₄ with these antisera. Further support came from the finding that the increasing T₄ content of the series of Tg preparations was not associated with such large increases in cross-reactivity with these T₃-specific sera as with the T₄-specific antiserum EB, where there was a very strong dependence of cross-reactivity on T₄ content. Cross-reactivity of these Tg preparations was not related to T₃ residue content (Table 1).

The four T₃-specific antisera could be divided into two groups on the basis of their relative dependence on T₄ content of Tg for binding energy. Sera ET and MM had 13- and 7-fold increases respectively in cross reactivity for a 130-fold increase in T₄ content, whereas for the same range cross-reactivity with GP and SC had 48- and 58-fold increases respectively. This presumably reflects relative differences in the importance of the iodothyronine and its surrounding features in the

Fig. 1. Interactions of human thyroglobulin preparations with two human iodothyronine-binding antisera. Displacement curves of ^{125}I -labelled iodothyronine binding to human autoantibodies in the presence of increasing quantities of competing ligands: $\text{Tg}[0.05]$ ($\square$), $\text{Tg}[0.3]$ ($\blacksquare$), $\text{Tg}[1.0]$ ($\blacktriangle$), $\text{Tg}[1.9]$ ($\circ$), $\text{Tg}[6.6]$ ($\bullet$), T_3 ($\triangle$), T_4 (∇). (a) T_3 -specific serum GP; (b) non-specific serum CK studied with $[^{125}\text{I}]\text{T}_4$ as tracer.

antigenic site when binding to the various immunoglobulins. The least observed dependence on T_4 would indicate the most dependence on neighbouring amino acids (e.g. ET and MM), and this is confirmed, since $\text{Tg}[0.05]$ had higher cross-reactivities with these two antisera than with the other two. Presumably, therefore, these antisera recognize different facets of the same antigenic site. The increase in reactivity as the T_4 content of Tg increases reflects the increased generation of the mature site due to the conformational changes in the neighbourhood as T_4 is synthesized. This may support the idea of the antibodies being generated against the iodinated protein.

Reactions with a non-specific iodothyronine antiserum

The pattern of cross-reactivities with the non-specific autoantibodies interact with thyro-ssembled that for the T_4 -specific serum EB. There was a very low cross-reaction with $\text{Tg}[0.05]$ containing little T_4 and a large increase in reactivity against both tracers when the T_4 content of Tg rose to an average of 1.9 residues/molecule followed by a smaller increase up to an average of 6.6 residues/molecule. The great dependence on the iodothyronine for binding indicates that in this case also the iodothyronine is an immunodominant feature of the antigenic site. Unlike the situation with antiserum EB, however, there was also a strong cross-reactivity with T_3 . The lack of discrimination between T_4 and T_3 suggests that the 5'-iodine atom is not important in interactions with this autoantiserum.

Conclusions

These results have confirmed experimentally our previous suggestions on the way in which T_3 -specific autoantibodies interact with thyroglobulin. A model may be described (Fig. 2) to illustrate how an antigenic site containing a T_4 residue reacts with antibodies of different specificities. The iodothyronine-binding properties of human autoantibodies against thyroglobulin appear to be related to the precise portion of the immunogenic region selected by the immune response in a particular individual. It is clear that it is the iodothyronine-containing region that is immunogenic and not the iodothyronine itself. Different portions of the region form the antigen for antibodies in different people.

The thyroxine residue may or may not be a dominant feature of the site. If dominant, the antibodies may exhibit a specificity for T_4 or be non-specific. If the thyroxine residue is not dominant and surrounding structures form the major determinants, then a mismatch with T_4 could be tolerated (and an accidental specificity for T_3 may exist), since sufficient binding energy can be generated with other

Fig. 2. Model for the different modes of binding by human autoantibodies to an hormonogenic site in Tg

In the upper part of the Figure T₄-specific immunoglobulin (T₄Ab) uses T₄ as a major determinant; in the lower part of the Figure the T₄ residue is a minor determinant for the T₃-specific immunoglobulin (T₃Ab).

amino acid side chains to register an adequate immune response. It is therefore unnecessary to postulate the presence of T₃ in the original immunogen which generated T₃-specific antibodies. The micro-specificity of each T₃ antibody examined may be different. If the individual binding sites can be more closely defined, then this type antibody will have value in the mapping and comparison of the hormonogenic regions in Tg from human and other species.

The question still remains as to why this region of Tg should be immunogenic. The protein is, after all, present at birth and from that time onwards in the circulation. Schneider *et al.* (1983) have shown

that circulating Tg is not iodinated, and thus spillage of iodinated Tg from the thyroid by some means could expose the individual to a new 'foreign' antigen. It is therefore conceivable that the conformational changes consequent upon synthesis of T₄ residues in Tg render the region immunogenic.

We thank Professor D. S. Munro, Dr. M. W. J. Davie and Dr. E. B. Mitchell for sending us some of the sera used in this study. We are most grateful to Mr. Keith Bullock for turning our ideas into the illustration in Fig. 2 and to Mr. E. W. Matthews and Mr. D. Fetherbe for the amino acid analyses.

References

- Byfield, P. G. H. & Bond, A. (1982) *Biochem. Soc. Trans.* **10**, 103
- Byfield, P. G. H., Bond, A., Copping, S. & Himsworth, R. L. (1982) *Biochem. J.* **207**, 471–478
- Byfield, P. G. H., Clingan, D. & Himsworth, R. L. (1984) *Biochem. J.* **219**, 405–410
- Hartley, B. S. (1970) *Biochem. J.* **119**, 805–822
- Himsworth, R. L., Byfield, P. G. H. & Copping, S. (1983) *Life Sci.* **32**, 119–125
- Kjeld, J. M., Kuku, S. F., Diamant, L., Fraser, T. R., Joplin, G. F. & Mashiter, K. (1975) *Clin. Chim. Acta* **61**, 381–389
- Lissitzky, S., Bismuth, J. & Rolland, M. (1962) *Clin. Chim. Acta* **7**, 183–189
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Marriq, C., Rolland, M. & Lissitzky, S. (1982) *EMBO. J.* **1**, 397–401
- Nye, L., Pontes de Carvalho, L. C. & Roitt, I. M. (1980) *Clin. Exp. Immunol.* **41**, 252–263
- Pearce, C. J., Byfield, P. G. H., Edmonds, C. J., Lalloz, M. R. A. & Himsworth, R. L. (1981) *Clin. Endocrinol.* **15**, 1–10
- Schneider, A. B., Ikekubo, K. & Kuma, K. (1983) *J. Clin. Endocrinol. Metab.* **57**, 1251–1256
- Von Arx, E. & Neher, R. (1963) *J. Chromatogr.* **12**, 329–341