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Thyroglobulin and Thyroglobulin Antibodies in the Serum of Normal Adults

E. S. K. Assem, W. R. Trotter and G. Belyavin

*Medical Unit, and Department of Bacteriology, University College Hospital Medical School, London, W.C.*1

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Summary. Sera from fifty-six adults (males and females) were tested for free thyroglobulin and anti-thyroglobulin antibodies by electrophoretic immunoretention and chromatographic techniques using ^{131}I-labelled thyroglobulin. The results were also compared with those previously reported in seventy-two parturient women and their newborn infants as well as three athyrotic cretins.

The results show that from one-half to two-thirds of normal non-pregnant females and from one-third to one-half of normal men have some material in their serum which binds labelled thyroglobulin and which may be anti-thyroglobulin antibody. A non-specific binding could not be excluded. The incidence of this phenomenon tends to have a positive correlation with age. On the other hand, one-fifth to one-third of newborn infants have 'free thyroglobulin'-like substance in their serum (0·005–0·05 μg/ml). This was also found in a significantly smaller proportion in adults but was a particularly rare finding in non-pregnant women.

The implications of these findings on current immunological concepts are discussed.

INTRODUCTION

In a previous report (Assem, 1964), where a radioimmunological method was used for the assay of thyroglobulin in serum, it was found that sera from normal adults showed a wide range of values for the ratio of free to bound labelled thyroglobulin. These results when compared with pooled normal adult serum to which standard amounts of human thyroglobulin were added, were compatible with one of two interpretations: (a) that about a quarter of all normal adults have free thyroglobulin in their serum or (b) that most normal adults have anti-thyroglobulin antibodies in their serum. The present report provides evidence for the latter interpretation, but the results are also compatible with the presence of free thyroglobulin in a minority of normal adult males, and in a significantly higher proportion of newborn infants.

METHODS AND MATERIAL

The human thyroglobulin used in our experiments (supplied by Messrs. Burroughs Wellcome & Co.) is ultracentrifugally homogeneous. Simple electrophoresis on cellulose acetate, which was used as a supporting medium in the electrophoretic immunoretention technique failed to demonstrate any heterogeneity. Although thyroglobulin can be shown to be heterogeneous in other respects (Assem, to be published) it behaved as a single protein when submitted to the techniques used in the present work.

^{131}I-labelled human thyroglobulin was used to detect both free thyroglobulin and anti-thyroglobulin antibodies in serum by two techniques: (a) electrophoretic immunoretention and (b) radioimmunochromatography on DEAE-cellulose. The first of these techniques has been described previously (Assem, 1964). The most reproducible results were obtained with an electric potential of 200 V at room temperature, for 4 hours, using 'veronal' buffer (0·07, pH 8·6), the sample being placed midway between the electrode vessels. The cellulose acetate strips were scanned for radioactivity in a Baird & Tatlock chromatogram scanner. In order to calculate the ratio of 'bound' to 'free' thyroglobulin, the strips were usually divided at a point 0·5 cm on the anodal side of the origin; or, when the thyroglobulin showed reduced electrophoretic mobility as a result of partial denaturation (though still immunologically active), at the origin. The results were checked by cutting the strips and counting them in a well-type scintillation counter. The anodal and cathodal peaks represent the free and the bound forms respectively. The second technique, radioimmunochromatography, was used to separate 'free thyroglobulin', which does not move from the start line, from soluble thyroglobulin–anti-thyroglobulin

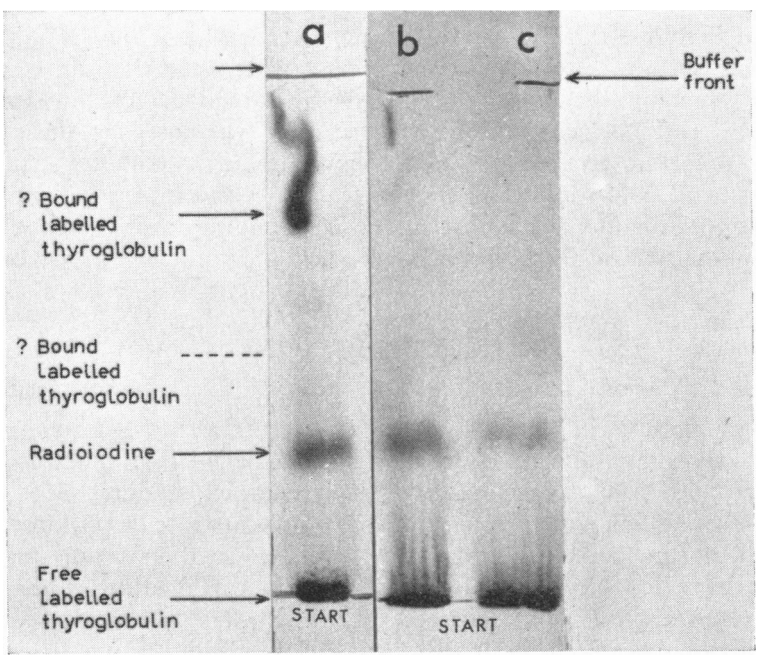

FIG. 1. Autoradiograms of DEAE-cellulose radioimmunochromatograms of labelled thyroglobulin added to normal human sera (a and b), and calf serum (c).

complexes which move with other serum proteins (Fig. 1). Untreated diethylaminoethyl-cellulose paper (DE 20, Whatman) and 0·5 M tris-phosphate buffer of pH 8·0 were used. The chromatography was done horizontally and the material subjected to the run was applied to the start line as soon as the buffer front reached 18 cm from the start line. The strip was finally dried and scanned for radioactivity. The ratio of bound/free labelled thyroglobulin was calculated from the proportions of radioactivity in the first peak (free thyroglobulin at the start line) and the remaining radioactive peak(s). ^{131}I-iodide which

is a contaminant of labelled thyroglobulin (1–5 per cent) forms a peak with an R_f value of 0·35.

Sera from ten normal adults were also applied to small columns of DEAE, in order that the ability of the γ-globulin fractions to deviate labelled thyroglobulin could be estimated in isolation from other serum components. Calf and guinea-pig serum was treated in the same way as a control. The conditions necessary for the elution of γ-globulin from DEAE were first worked out on a larger similar column by measurement of protein content, osmolarity and pH in serial samples with confirmation of the purity of the γ-globulin fraction by paper electrophoresis. 0·2 ml of serum was applied to the small (0·3 × 5 cm) columns, and the γ-globulin eluted with 1 ml of 0·1 M tris-phosphoric acid buffer at pH 8·0. The results were expressed as the percentage of labelled thyroglobulin bound by the γ-globulin fraction of the test serum, divided by the percentage bound by the γ-globulin fraction of the control serum (sample/standard ratio), when tested by the electrophoretic method.

The sera. Single samples of sera (sent to the laboratory for Wasserman tests) were obtained from twenty-eight non-pregnant women and twenty-three men. Sera were also obtained from five males at weekly intervals, for 8 weeks from three, for 7 weeks from one and for 4 weeks from the last one. Calf serum (Oxoid) and 2 per cent bovine plasma albumin (Armour) in saline were used as a control in each run. Guinea-pig and horse sera were also used in a few runs. Sera from three athyrotic cretins were also investigated.

RESULTS

ELECTROPHORETIC METHOD

When ^{131}I-labelled thyroglobulin was subjected to electrophoresis in the presence of calf serum, guinea-pig serum or bovine plasma albumin, from 75 to 90 per cent of the total radioactivity migrated as an anodal peak; the remaining proportion (10–25 per cent) remained in the cathodal region, behaving in this respect as if it were antibody bound. The factors determining this behaviour are uncertain but it is possibly associated with non-specific absorption of the labelled material to serum proteins or to the supporting medium. The actual proportion on the cathodal side of and including the start line varied in different experiments using different batches of labelled thyroglobulin, possibly because partial denaturation had occurred in some of the thyroglobulin in calf serum. Results were best expressed as the ratio of the percentage of radioactivity bound by the test serum to the percentage bound by the sample of calf serum (sample/standard ratio).

More than half the adult human sera tested bound ^{131}I-labelled thyroglobulin to a greater extent than calf serum. As can be seen from Fig. 2, the addition of an excess of unlabelled thyroglobulin reduced the proportion of labelled material moving to the cathode to approximately the control level, obtained with calf serum. This provided presumptive evidence that the excess binding of ^{131}I-labelled thyroglobulin by human as compared with calf serum could be attributed to the presence in the human sera of an antibody to thyroglobulin. Table 1 also shows that in ten of the sera from normal adults, the γ-globulin fractions showed about the same ability to bind labelled thyroglobulin as the whole sera from which they were derived. It was of some interest that the serum of three presumed athyrotic cretins bound no more labelled thyroglobulin than calf serum.

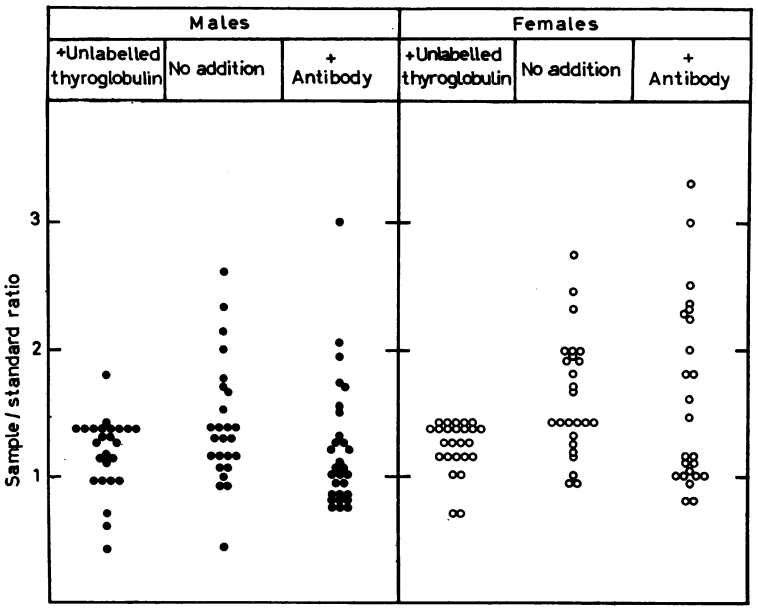

FIG. 2. Normal adults' sera tested by the electrophoretic immunoretention technique. Each serum was tested for its ability to alter the apparently 'bound' proportion of labelled thyroglobulin, by three varieties of runs: (a) with no addition, (b) with the addition of 5 μg unlabelled thyroglobulin to 0·1 ml serum, and (c) with the addition of enough dilute Hashimoto serum to give a bound/free ratio of just over 2/1. The results have been expressed as the ratio of the 'bound' proportion of labelled thyroglobulin, in each variety of run of human serum, to that of control calf serum with the same additions (sample/standard ratio).

TABLE 1

SAMPLE/STANDARD RATIOS OF THE γ-GLOBULIN FRACTION OF TEN NORMAL ADULT SERA, COMPARED WITH THE SAMPLE/STANDARD RATIOS OF WHOLE SERUM FROM THE SAME SUBJECTS

Subject No.	Sample/standard whole serum	Sample/standard ratio of γ-globulin fraction	T.R.C. titre
1	0·8	1·1	1/8
2	0·7	1·1	1/2
3	0·8	1·2	1/8
4	0·95	1·2	1/2
5	1·0	1·0	–ve
6	2·0	1·3	1/32
7	—	1·6	1/32
8	1·3	1·3	1/32
9	1·4	1·3	1/32
10	2·0	1·8	1/64

On the other hand, a small proportion of normal sera bound a proportion of labelled thyroglobulin equal to that of the control calf serum, but when dilute Hashimoto serum was added, the proportions of labelled thyroglobulin in the 'bound' form were lower in the presence of those sera than in the control calf serum. This was interpreted to mean the presence of 'free' thyroglobulin, in the serum of these individuals. Decreased binding

which could be interpreted in this way was, however, found only in about 10 per cent of the males, and none of the females. However 9 per cent of sera from women at term and 18 per cent of newborn infants' sera, behaved as if they contained small amounts of free thyroglobulin.

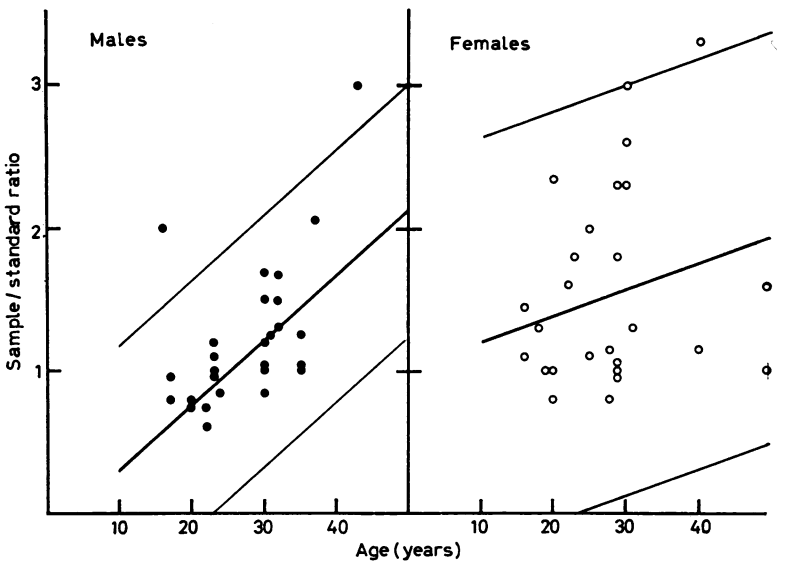

FIG. 3. Relationship between age and thyroglobulin-binding capacity of serum obtained as in Fig. 2 (with the addition of dilute Hashimoto serum).

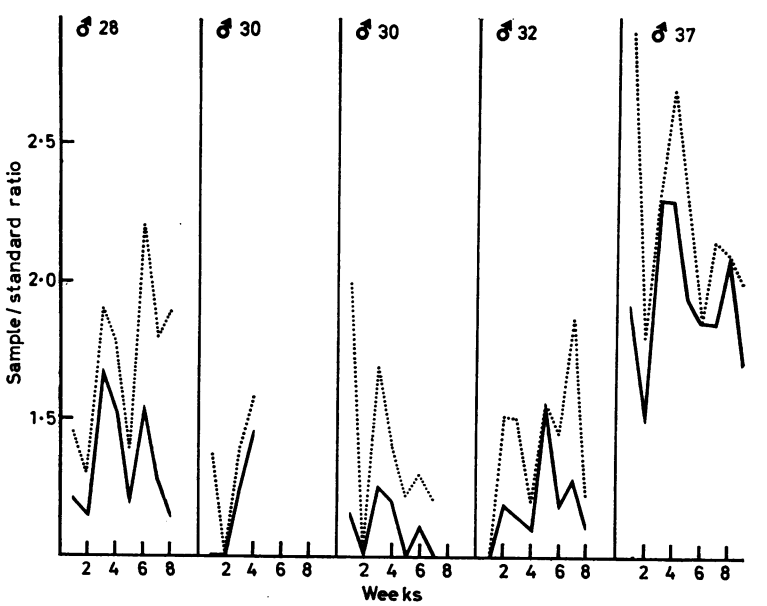

FIG. 4. Serial assays in five normal adults on weekly samples of serum. ·····, No addition; ——, with unlabelled thyroglobulin. Sample/standard ratio as in Fig. 2.

With the exception of the few male subjects mentioned above, the results obtained in normal adults could be interpreted in terms of a range of antibody concentrations. It can be seen from Fig. 3 that the amount of apparent antibody present showed some relationship to sex and age. Female sera contained significantly more than male, but the males showed a clearer tendency for antibody content to increase with age.

Week-to-week variation in thyroglobulin binding capacity was examined in five normal subjects. The degree of variation observed was significantly greater than the error on repeated estimation of single samples (Fig. 4). There was no indication of any regular periodicity.

CHROMATOGRAPHIC METHOD

In the electrophoretic method, any labelled thyroglobulin which is antibody-bound remains at or near the origin. In the chromatographic method the antibody-bound thyroglobulin migrates, if it remains in a soluble form, whereas free thyroglobulin does not move from the origin. Hence the use of both techniques on the same set of samples provided some protection against artefacts. In general, the results of chromatographic analysis were less reproducible than those of the other method.

When ^{131}I-labelled thyroglobulin was added to calf serum or bovine plasma albumin, about 5 per cent migrated. This proportion was much greater (up to 40 per cent) in many of the sera from normal adults. In order to take into account variations from one experiment to another the results were again expressed as the ratio of the percentage migrating in the test serum, to the percentage migrating in the calf serum control, run simultaneously (sample/standard ratio). When the results of electrophoresis and chromatography were compared, a high degree of correlation ($r = +0{\cdot}67$) was evident (Fig. 5).

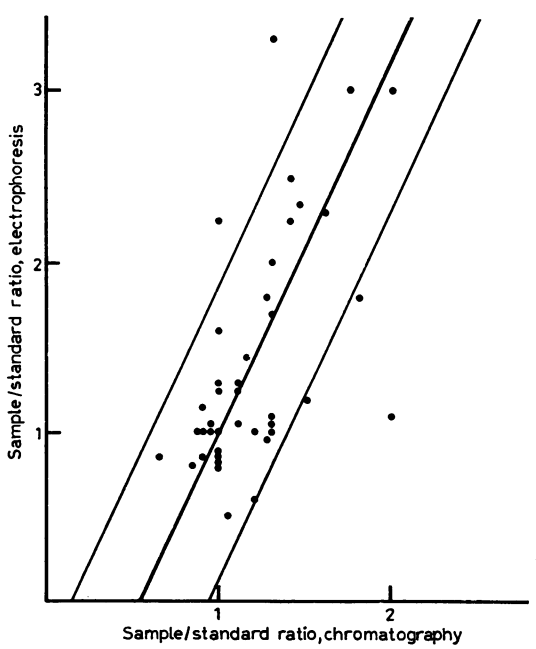

FIG. 5. Correlation between electrophoretic immunoretention and immunochromatography. Sample/standard ratio as in Fig. 2 (with the addition of dilute Hashimoto serum).

The results have been summarized in Table 2.

TABLE 2

Source	No. of sera	% of sera binding labelled thyroglobulin in excess of calf and guinea-pig sera and BSA (? anti-thyroglobulin antibodies)		% of sera binding labelled thyroglobulin within 2 SD of controls		% of sera inhibiting the binding of labelled thyroglobulin by dilute Hashimoto serum (? free thyroglobulin)	
		Electro-phoresis	Chromato-graphy	Electro-phoresis	Chromato-graphy	Electro-phoresis	Chromato-graphy
Normal men	28 (25)+	39 (32–46) (16)+	34 (20–48)	51 (44–58) (60)+	56 (47–65)	10 (24)+	10
Non-pregnant women	28 (25)+	56 (44–68) (28)+	64 (60–68)	44 (32–56) (56)+	36 (32–40)	0 (16)+	0
Women at delivery	(72)+	(12)+		(59)+		(29)+	
New-born infants	(72)+	(12)+		(55)+		(33)+	

% given as mean with range in brackets.

()+ % when compared with standards made of pooled human adults' serum.

DISCUSSION

Taken at their face value, the present results indicate that the serum of more than half the normal adult population contains antibody to thyroglobulin. As this proportion is a good deal larger than any previously reported by workers using the tanned red cell agglutination test, the evidence that binding of labelled thyroglobulin really indicates the presence of a specific antibody needs to be critically reviewed.

The good agreement between the electrophoretic and chromatographic methods seems to provide the best evidence that the observed deviations of labelled thyroglobulin represented the effect of a specific property of the serum under test. This at least excluded the obvious artefact of adherence of the labelled material to the supporting medium, since antibody-bound material remains at the origin in the first method and free thyroglobulin migrates, whereas exactly the reverse happens in the second method.

Artefacts due to non-specific binding to serum proteins cannot be so readily excluded. Indeed the fact that small proportions of the labelled material no longer behave as 'free' thyroglobulin when tested in calf serum or bovine plasma albumin shows clearly that such non-specific binding can occur. If it could be assumed that the proteins of calf serum were in this respect identical with those of human serum, then the excess of binding observed with most of the human sera could not be attributed to this type of non-specific effect. Unfortunately, this assumption cannot be justified and it has to be admitted that calf serum does not provide the perfect control. Serum from authentic human cases of athyrotic cretinism would be more satisfactory, since the serum proteins are not likely to differ (after control of hypothyroidism) from those of normal subjects, and as they never possessed a thyroid gland it would seem unlikely that they could form antibodies against thyroglobulin. Such cases are not sufficiently available to provide a practicable control, but it is interesting that the three cases in the present series behaved as expected.

Further support for the view that the excessive binding of labelled thyroglobulin was an expression of the presence of a specific antibody came from the observation that the addition of stable thyroglobulin (10–100 μg/ml of serum) reduced the proportion bound by

all sera to about the same level as the calf serum control. This result is the more significant in that the addition of stable thyroglobulin in the same amounts did not affect the proportion of labelled material bound non-specifically to calf serum.

Some further support for the antibody hypothesis was provided by the finding of tanned red cell agglutination titres of 1/32–1/64 in five sera with high binding activity. However, among the remaining sera there was no apparent correlation between tanned red cell titre and binding activity.

It could moreover be shown, by separation of ten of the sera on DEAE-cellulose columns, that the factor which bound labelled thyroglobulin was contained in the γ-globulin fraction. This gives further support to the idea that the observed differences in thyroglobulin-binding capacity are indeed reflections of varying antibody content.

If this conclusion is accepted, then it follows that the occurrence of antibodies against thyroglobulin in human sera must be regarded as a 'normal' phenomenon. Although the formation of antibodies against self constituents is now widely accepted in pathological states, the idea that the same process might be a normal physiological event is more difficult to assimilate. There are, however, at least four other recorded instances in which there is good reason to suppose that autoantibody formation had occurred in normal animals: (i) Heimer, Levin and Rudd (1963) found a globulin resembling 'rheumatoid factor' in the serum of many normal subjects over the age of 65; (ii) Walsh (1925) found natural spermotoxin in guinea-pig serum; (iii) Kidd and Friedewald (1942) described antibodies in the serum of adult rabbits to a sedimentable constituent of many tissues; (iv) Boyden (1964) using a different technique found antibodies against autologous and homologous skin extracts in adult rabbits. In addition there is evidence to suggest that by the immunofluorescent technique it may be possible to demonstrate the affinity of the γ-globulin of normal animals to autologous tissue, e.g. Allerand and Yahr (1964) have demonstrated the affinity of the 7S γ-globulin for tissue of the central nervous system in monkey as well as in human beings.

Recent work by Daniels, Pratt, Roitt and Torrigiani (1964, personal communication) has shown that in some primates thyroglobulin may be continually 'leaking' from the lymph channels draining the thyroid into the blood. If thyroglobulin is entering the human circulation in the same way, then most normal people would be expected to show evidence of circulating antibody to it. The only exceptions would be those who had become immunologically tolerant to it, from contact in uterine life. A proportion between one-fifth and one-third of newborn infants may, on one interpretation of the present results, have some free thyroglobulin in their serum, but one can at present only speculate as to whether these are the individuals who in later life will fail to show any evidence of antibody to thyroglobulin.

The evidence that any normal subjects (adult or newborn) do in fact have free thyroglobulin in their serum is more difficult to interpret than that relating to antibodies. All that can be safely said is that the evidence is compatible with the view that free thyroglobulin circulated in quite a large proportion of the newborn, but had disappeared from the serum of most subjects by the time adult life was reached. The alternative interpretation, purely in terms of antibody, is also defensible. This would assume that the non-specific capacity of human serum (if it could be wholly freed of antibody) to bind labelled thyroglobulin is less than that of calf serum. Those sera which appear to contain free thyroglobulin would then be regarded as the only ones from which antibody to thyroglobulin was entirely absent.

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