SEX AND AGE CORRELATED REFERENCE VALUES OF SERUM THYROGLOBULIN MEASURED BY A MODIFIED RADIOIMMUNOASSAY

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ABSTRACT

The aim of the present investigation was to describe variations in serum thyroglobulin in relation to sex and age in a group of normal persons. The method used was a modified double antibody radioimmunoassay characterized by pre-incubation at 37°C of standard or sample with antiserum, resulting in a reduced total incubation time. Both sensitivity and precision were comparable to other published methods.

Of the 152 blood-donors initially investigated, 7 were excluded due to the presence of antithyroglobulin antibodies as evidenced by a radioassay. Both sexes were equally represented with an even distribution of ages from 20–65 years.

Increased serum thyroglobulin with increasing age was demonstrated, the correlation being significant in women (Kendall’s τ, P < 0.001). Detectable concentrations of serum thyroglobulin (above 1.7 µg/l) were found in 94.9%. Based on the logarithmic transformation, the upper reference limits were determined for men ≤ 40 years: 36 µg/l, > 40 years: 44 µg/l (difference between groups not significant, P > 0.05), and for women ≤ 40 years: 30 µg/l, > 40 years: 60 µg/l (significant difference, P < 0.005).

Since the middle of the sixties the presence of thyroglobulin (Tg) in human serum has been well established (Hjort 1961, 1963; Hjort et al. 1970). Recently specific radioimmunoassays for the measurement of Tg have been developed (Roitt & Torrigiani 1967; Van Herle et al. 1973; Ochi et al. 1975; Schneider et al. 1977; Pinchera et al. 1977; Bodlaender et al. 1978).
This paper presents a rapid method for measurement of serum Tg, including a well-defined reference sample group, giving reference intervals according to sex and age. Furthermore the possible contribution of short-term intra-individual variation to the width of the reference intervals has been investigated (Cotlove et al. 1970).

MATERIAL AND METHODS

Material

None of the subjects included in the study has previously had any endocrinological disorders. They received no medication at the time of investigation (P-pills included), nor had they been subjected to X-ray investigations within the past 3 months. Body weights were within normal limits related to their heights. Serum concentrations of TSH, triiodothyronine and thyroxine were all within reference limits. All specimens with anti-Tg autoantibodies (TgAb) were excluded (see below).

Initially 152 blood-donors were investigated. Based on questioning and inspection, no goitre was present in any of the persons. Seven were excluded because of the presence of TgAb, leaving 145 for the study. Of these 72 were women (age: 20–68, median years), and 73 were men (age: 19–68, median 39 years). Blood specimens were drawn in a supine position. Samples collected during the day were centrifugated in the afternoon. Serum was immediately separated and stored at −20°C.

Intra-individual variation was studied in 10 male volunteers with no palpable goitre. Blood was sampled on 3 consecutive days between 7.30 and 9.00 a.m. after an overnight fast. The subjects were supine for 10–15 min before venipuncture with a minimum of tourniquet. After removal of the tourniquet, a maximum of 50 ml of blood was collected through the suction effect of a syringe.

All specimens from the same individual were run in the same assay. Specimens from the normal persons were run at random in relation to age of the persons in a limited number of assays.

Methods

Measurement of thyroglobulin. – The method used was a modification of the double antibody radioimmunoassay described by Van Herle et al. (1973). Tg was purified and iodinated as described previously (Feldt-Rasmussen 1978), and antiserum raised in rabbits as described by Harboe & Ingild (1973). The standard was kept at −80°C in phosphate buffered saline (PBS: 0.0035 m phosphate in 0.15 m NaCl, pH 7.0, 0.02% NaN₃) with the addition of 5% (v/v) of human serum (0-serum) containing unmeasurable levels of Tg and TgAb. The [¹²⁵I]Tg was controlled by ultracentrifugation (Feldt-Rasmussen 1978). The standard curve was constructed using dilution of the standard in 0-serum (1:5 in PBS). Non-specific binding was hence tested with addition of 0-serum. Two hundred µl of standards or serum samples were pre-incubated (37°C for 4 h) in polystyrene tubes (7 × 11 mm, Nunc, Denmark) with 100 µl rabbit antithyroglobulin (diluted, 1:10 000 in PBS; final dilution 1:70 000). Further incubation at room-temperature overnight was performed after addition of 100 µl (i.e. approximately 0.3 ng) ¹²⁵I-labelled Tg in PBS with addition of EDTA Na (5 g/l) (corresponding to 10 000 cpm). Thereafter 100 µl rabbit serum (Burroughs Wellcome) diluted 1:400 with PBS and 200 µl anti-rabbit immunoglobulins from swine (Dako, Denmark, code 21-090) diluted 1:10 in PBS were added successively and tubes further incubated at 37°C for
The precipitates were collected by centrifugation for 15 min., and electrophoresis was carried out at 100 V (constant voltage) for 19 h in a gel electrophoresis apparatus GE-4 II® from Pharmacia (Sweden). The gel was stained in amido black in 7% acetic acid and subsequently destained in 7% acetic acid.

**Protein determination.** - For the Tg-standard, protein concentration was determined by a modification of the Biuret method (Doumas 1975), as well as by dry weight determination after extensive dialysis against distilled water (Hunter 1966).

**Measurement of thyroglobulin autoantibodies.** - The concentrations of TgAb in serum were determined by a radioassay using [125I]Tg and co-precipitation with antihuman IgG (Dako, Denmark). a.m. Salabé et al. (1972, 1974) with the following modifications:

- A reference serum, calibrated against the Medical Research Council research standard A 65/73, by definition containing 1 M unit/l (mU/l), was serially diluted and analysed, giving a nearly rectilinear logit-log standard curve, which was used for calculation of the antibody content of the similarly diluted samples for assay.
- The method correlated well to the antigen binding capacity procedure used by Salabé et al. (1974). R = 0.997 (our method = 0.019 × antigen binding capacity – 0.33).
- The mean coefficients of variation in the concentration range 0.74 to 241 mU/l were 3% (within-assay) and 8% (total-assay), respectively.
- The detection limit calculated from mean blank + 3 × SD was < 0.001 mU/l, but as samples at this low level often exhibited dilution curves not parallel to the standard, a limit of 0.002 mU/l was somewhat arbitrarily chosen.

**Interference of TgAb in the Tg method.** - To evaluate the practical consequences of the interference of TgAb, when measuring Tg in serum specimens, 5 serial dilutions of a patient serum with a TgAb concentration of 5 mU/l were made. To 400 µl of these dilutions, 400 µg Tg (16 mg/l) was added. The combination of Tg and the various antiscrum dilutions corresponded to 2.5 and 5 times Tg excess and 2.5, 12.5, and 25 times TgAb excess, respectively. Each tube was serially diluted from 10 to 1000 times and the final concentrations of Tg and TgAb were measured in these dilutions.

**Other methods used for quantitation of serum constituents.** - Serum TSH was measured by RIA-gnost® hTSH (Behringwerke AG). serum thyroxine by a competitive protein binding method (Tetrasorb® 125-T1 Diagnostic Kit, Abbot) and serum triiodothyronine by radioimmunoassay (T3RIA Kit II®, Dainabot Radioisotope Laboratory).
RESULTS

Method for measurement of serum Tg

Tg-standard. – The purity of the Tg-standard was, apart from previously published immune electrophoretic procedures (Feldt-Rasmussen 1978), further evaluated by PAAE. One band corresponding to a molecular weight of approximately 660,000 was seen.

The protein concentration of the Tg-standard agreed within 5 % between the two method used.

Choice of assay conditions. – By varying pre-incubation from 1/2 to 6 h, an optimal precision around 4 h was found. The incubation with a second antibody was found independent of time within 1/2 to 2 h. Increasing temperature of the reacting medium during these two incubation steps, increased the slope of the standard curve.

A typical example of the unweighted and weighted logit-log curves is shown in Fig. 1. The mean counts in zero tubes were in this example 14,920 counts/10 min, corresponding to a fraction bound at zero concentration (B_/T) of 16 %. Five independent assays yielded a mean correlation coefficient of 0.97.

Precision. – The reproducibility of the assay was evaluated using duplicate or more determinations of three control sera at various Tg-concentrations over a 3 week period (9 assays). The mean Tg-concentrations of the pools were 4.2, 9.5 and 28.9 μg/l. The within-assay coefficients of variation were 3.6 % (n = 18), 8 % (n = 26), and 8 % (n = 29), and the total-assay coefficients of variation were 11, 15 and 14 %, respectively.

![Fig. 1.](image_url)

Standard curve of serum thyroglobulin standard points: * in weighted and unweighted logit-plots. The weighted curve was in all instances used for calculation of unknowns.
Sensitivity. – The least detectable concentration was determined, using the calculated error at zero concentration. Two \( \times \) SD was subtracted from mean counts at zero point, and the corresponding Tg-concentration read off the standard curve. In 10 assays it ranged between 1.5–2.0 \( \mu \)g/l (median 1.7 \( \mu \)g/l).

Recovery and dilution experiments. – By adding 100 \( \mu \)l Tg-standard (220 \( \mu \)g/l) to 990 \( \mu \)l of a serum sample containing 5 \( \mu \)g Tg/l a theoretical Tg-concentration of 27 \( \mu \)g/l would be expected. In 8 experiments using 2 to 4 determinations in each, a mean Tg-concentration of 29 ± 1.6 \( \mu \)g/l (SEM) was obtained corresponding to a mean recovery of 105 ± 6 \% (SEM).

The accuracy was further evaluated by diluting 57 randomly selected specimens (\( \bar{x} \): 40 \( \mu \)g/l; range: 5.4–130 \( \mu \)g/l) up to four times, in 0-serum and running them in the assay, giving a mean of 98 ± 2.2 \% (SEM) of calculated concentrations.

Specificity. – Addition to the 0-serum of up to non-physiologically high concentrations (0.5 g/l) of 3-iodo-L-tyrosine, 3,5-diiodo-L-tyrosine, 3,3',5-triiodo-L-thyronine and 3,3',5,5'-tetraiodo-L-thyronine (Sigma, St. Louis) instead of standard Tg, gave results not significantly different from 0-serum (i.e. undetectable). Specificity was further evidenced by removal of Tg from a serum specimen by affinity chromatography with Tg-antibody coupled to CNBr-Sepharose 4B® (Pharmacia, Sweden), yielding unmeasurable serum Tg.

Interference of TgAb in the Tg method. – By adding Tg-standard to TgAb containing serum samples the dilution curves of the Tg-TgAb in antigen excess coincided with the standard curve within analytical error. The corresponding measurements of TgAb showed unmeasurable concentrations. In-
Table 1.
Non-parametric and parametric percentile-calculations of serum Tg (µg/l) in selected blood-donors subdivided with respect to sex and age. 95% reference intervals of each age group are indicated in italics.

<table>
<thead>
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<th>Number</th>
<th>Non-parametric</th>
<th>Log Gaussian</th>
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<td></td>
<td>Percentiles</td>
<td>Value of</td>
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<tr>
<td></td>
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<tr>
<td>Women&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
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<td>\leq 40&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup> Significant difference between groups: \( P < 0.001 \) (non-parametric), \( P < 0.005 \) (t-test).

<sup>b</sup> No significant difference between groups (\( P > 0.05 \)).

<sup>c</sup> Within none of the age groups were there any significant difference between men and women.

<sup>d</sup> Not calculable for sample sizes below 40.

<sup>*</sup> \( P < 0.05 \).
creasing the antibody excess gave progressively deviating parallelity from the standard curve and decreasing quantitation of Tg. Increasing amounts of TgAb added to a sample with 11 µg/l resulted in detectable levels of TgAb at the point of approximately 30% reduction of the measured Tg concentration. For higher Tg-concentrations, TgAb was detectable, before reduction of the measured Tg was significant.

Reference intervals of Tg

The results of measurements of serum Tg in relation to age of healthy men and women are shown in Fig. 2. Of 145 persons 136 (94%) had detectable levels of serum Tg. There was a tendency towards higher levels with increasing age. This was significant in women (Kendall's \( \tau = 0.264, P < 0.001 \)) but not in men (\( P > 0.10 \)).

To test this hypothesis further, and to quantitate this dependence, the material was divided into groups of 10 years intervals for both men and women, and the results were calculated by both parametric (log Gauss) and non-parametric methods. Sera with undetectable levels of Tg were arbitrarily given the value 1.0 µg/l. Increasing 50-percentiles with increasing age were noted in both sexes and for women the concentration increased significantly (approximately 100% ) just around 40 years. For men the increase was less (approximately 50% ) and not significant at the 5% level. This justified division

![Fig. 3.](image)

Distribution of serum Tg-values in relation to sex and age (probit-log scale). Each point indicates the percentage of persons (Y-axis) with a serum Tg-level below the value read off the abscissa. ○ \( \varphi \leq 40 \) years; ● \( \varphi > 40 \) years; □ \( \varnothing \leq 40 \) years; ■ \( \varnothing > 40 \) years.
of the material around 40 years. As shown in Table 1 only some of these groups were actually distributed in a log Gaussian way. The distributions showed in no instances kurtosis, but for the groups consisting of women > 40 years and the total group of men, respectively, there was a certain degree of skewness ($P < 0.05$).

The reference intervals for men and women were given by the 2.5 and 97.5 percentiles of the log Gauss calculations in Table 1.

The distributions of Tg-values for the above mentioned 4 samples groups are illustrated on probit-log scale in Fig. 3.

**Short-term intra-subject variability of serum Tg**

All values from the 10 subjects included were measurable and below the upper reference limit for men ($< 40$ years) (Table 1). The mean serum Tg for the 10 subjects was $7.3 \mu g/l$ and the calculated pooled standard deviation within individuals was $1.3 \mu g/l$, resulting in a total coefficient of variation (CV) of 18%. A control pool with approximately the same mean value ($6.8 \mu g/l$) estimated the analytical with-assay error to 11% (degrees of freedom: 30). From this the isolated CV was calculated to be 14% (i.e. excluding the analytical error).

**DISCUSSION**

The precision of the method presented is comparable to that of others (Schneider et al. 1977; Pinchera et al. 1977; Van Herle et al. 1973). The advantage of the present method is its speed obtained largely by pre-incubation of sample and antiserum at higher temperature, as recently also shown by Bodlaender et al. (1978).

The lack of international preparations of standard and control material made inter-laboratory comparison of accuracy impossible, the consequence of which have been discussed by Bangham & Cotes (1974). Therefore, the above control procedures employed for Tg were important for the specificity in relation to the antigen. The high specificity in relation to the rabbit antiserum used was indicated by excluding interference from even non-physiologically high levels of thyroid hormones, as also done by other investigators (Van Herle et al. 1973; Schneider et al. 1977; Pinchera et al. 1977).

The importance of TgAb as an interfering substance in the methods has been thoroughly investigated by Schneider & Pervos (1978). The second precipitating antibody against rabbit immunoglobulin used in the present method showed cross-reaction to human IgG, whereby TgAb in moderate concentrations would reduce the quantitative results of serum Tg (Schneider & Pervos 1978). Our studies showed that the quantitative interference of autoantibodies
was dependent also on the ratio between Tg and TgAb. The radiolabelled method for measurement of TgAb had a sensitivity sufficient to reveal the presence of TgAb at a level corresponding to the one causing slight reduction in the results of a serum Tg concentration of 11 µg/l. This implied that serum with unmeasurable TgAb, giving Tg-results above this limit were nearly free from interference from TgAb. It did not imply, however, that TgAb could not be present, as Tg-TgAb complexes in Tg excess would be quantified like the free Tg.

Tg-values at or below approximately 20 µg/l, on the other hand, might be biased by the presence of small amounts of TgAb. There were, however, no practical consequences of this, as this level was well below the upper reference limits, and only increased levels of Tg have been reported to be of clinical interest in e. g. follow-up of patients treated for thyroid cancer (Van Herle & Uller 1975; Lo Gerfo et al. 1977) and prediction of relapse of thyrotoxicosis in Graves' disease (Uller & Van Herle 1978).

The sensitivity of the present method was in terms of µg Tg/l of the same order as reported by some investigators (Schneider et al. 1977; Pinchera et al. 1977; Van Herle et al. 1973; Izumi & Larsen 1978). The percentage of normal persons with measurable concentrations of serum Tg differed between methods, that of the present paper (94 %) being comparable to results given by Izumi & Larsen (1978) and Pezzino et al. (1978).

Previously Ochi et al. (1975) reported that there was no difference between Tg-values in men and women. Only Van Herle et al. (1973) investigated both sex and age dependence of Tg-values and found no relation to age, but a sex difference. Our results showed a tendency towards higher Tg-values in both men and women related to age (Figs. 2 and 3), but it was significant only for women. The reason for not observing this effect previously might be due to differences in selection of the donor material and in the size of the group above 40 years of age (Torrigiani et al. 1969).

In the present investigation an even distribution of the persons in age and sex groups increased the significance of testing the distributions and differences. All groups tested, showed 10, 50 and 90 percentiles of almost equal value in both methods of calculation. Due to the relatively small sample sizes, the parametric description of the 95 % reference interval was superior to the non-parametric, reducing the confidence interval for the 97.5 percentile (Bliss 1967; Wulff 1976; Diem & Lentner 1970).

The variability of serum Tg within the same subject with time has previously been shown by Van Herle et al. (1973) for 4 normal persons to be within the reference range of 95 blood-donors. In our study normal males varied only to a small degree over a 3-day period, which information was important when evaluating changes in serum Tg over a short period as for instance for turnover studies following thyroid surgery (Feldt-Rasmussen et al. 1978).
Due to a higher frequency of thyroid disorders among women of the older age group (Werner & Ingbar 1971), and the potential risk of misclassifying some patients by comparing their results to a too narrow reference interval blood-donors (Ransohoff & Feinstein 1978), the findings in the present investigation of age and sex dependent serum Tg-values has urged the need for the use of matched control groups for future studies of serum Tg in patients with thyroid disease. This together with ensurance of the long-term stability of the method giving equal analytical circumstances comparing the reference population with patients (Dybkjær 1973; Grasbäck 1977) is of particular importance for a substance not internationally standardized.

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