T₃ releasing activity by Graves’ sera from Graves’ thyroid in vitro

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Abstract. The insensitivity of Graves’ thyroid to stimulation of cAMP formation by TSH as well as Graves’ immunoglobulins in vitro is well known. The present study was performed to find out Graves’ sera which may induce a final activation i.e. stimulation of T₃ release in Graves’ thyroid slices despite this insensitivity of tissue and to characterize determinants responsible for the efficiency of those sera.

Out of 20 sera from patients with active untreated Graves’ disease 6 were found to stimulate T₃ release from Graves’ thyroid in vitro. These 6 sera were effective in stimulating different Graves’ glands, irrespective of pretreatment with propranolol, thiama-zole (methi-mazole) or thiamazole plus iodine. In contrast, a significant response to bTSH was not observed in any Graves’ gland. For comparison, 17/20 of the same sera were able to stimulate T₃ release when tested on human goitrous thyroid. Sera which stimulated Graves’ slices revealed no higher stimulating activities in goitrous tissue than serum samples which did not. All sera were additionally assessed for TSH binding inhibiting immunoglobulins in a radioreceptor assay. Remarkably, Graves’ thyroid stimulating sera had a low or absent TSH binding inhibiting activity.

Thus, hormone release from Graves’ thyroid in vitro – in contrast to that from goitrous tissue – could only be activated by a minority of Graves’ sera. These Graves’ thyroid stimulating sera could be characterized to contain a selected spectrum of biologically active antibodies with a high TSH agonistic potency stimulating in the presence of a negligible TSH binding inhibiting activity. We conclude the qualitative composition of the antibody spectrum in the individual sera, such as the occurrence of so-called ‘TSH superagonists’, rather than the height of antibody titre as determined by various methods seems to be relevant for their Graves’ thyroid stimulating potency.

It is now generally accepted that autoantibodies which mimic the action of TSH cause hyperthyroidism in Graves’ disease. Antibodies of the IgG class have been shown to inhibit the binding of labelled TSH to thyroid membrane preparations (Smith & Hall 1974), to stimulate adenylate cyclase activity (Onaya et al. 1973) and to release T₃ from thyroid slices (Atkinson & Kendall-Taylor 1981; Laurberg & Weeke 1975).

However, there is only little direct evidence that these immunoglobulins are potent stimulators of Graves’ thyroid in vitro (Kuzuya et al. 1980; Kasagi et al. 1980). Moreover, an insensitivity of Graves’ tissue to stimulation by TSH as well as by thyroid stimulating immunoglobulins has been well documented (Holmes et al. 1978; Kuzuya et al. 1980).

The mechanisms leading to this unresponsiveness of Graves’ tissue are at present not fully understood, although various influences such as saturation of receptor with specific IgG, receptor down-regulation, decrease of substrates under experimental conditions and pretreatment of patients with antithyroid drugs and iodine have been discussed (Kuzuya et al. 1980; Kasagi et al. 1980; Shewring & Smith 1982; Onaya et al. 1973; Rapoport et al. 1976; Sherwin & Tong 1975; van Sande

The aim of the present study was to find out whether Graves’ glands which have been stimulated by endogenous autoantibodies are definitively unresponsive to Graves’ sera in vitro or whether the functional stimulation depends on the potency of sera added. For this purpose a T3 releasing bioassay (Atkinson & Kendall-Taylor 1981) was used, which reflects the final biological response. The determination of hormone secretion instead of cAMP accumulation appeared preferable, particularly since alternative pathways in transducing hormonal signals to the well established cAMP system have been described (Nishizuka et al. 1984).

Indeed, we found some Graves’ sera stimulating homologous Graves’ gland in vitro. In an effort to characterize these Graves’ thyroid stimulating sera more in detail, their T3 stimulating potency in human goitrous thyroid tissue and their TSH binding inhibiting activity in a radioreceptor assay were determined. The Graves’ thyroid stimulating capacity could be related to sera containing antibodies with a high degree of TSH agonistic potency i.e. high stimulatory activity occurring even in the absence of any detectable TSH binding inhibiting activity.

Materials and Methods

Thyroid tissue was collected at surgery from 8 patients with non-toxic diffuse goitre living in an area of endemic goitre (42 ± 10 years mean age) and from 10 patients with recurrent Graves’ disease after 15–24 month antithyroid drug treatment (45 ± 17 years). Two patients with Graves’ disease had been preoperatively treated with thiamazole (40–60 mg/day for 3–4 weeks) and 6 patients with thiamazole (40–60 mg/day for 3–4 weeks) and iodine (100 mg/day for 8 days) to restore euthyroidism. Two patients with mild hyperthyroidism (T4 165 and 173 nmol/l; T3 3.5 and 4.0 nmol/l; TBI 0.88 and 0.90) only received propranolol (3 × 40 mg/day for 8 days).

Sera were obtained from 20 other patients with active Graves’ disease before treatment. In all subjects included in this study diagnosis of Graves’ disease was confirmed by clinical observation, 99m technetium scintigraphy, hormone analysis (T4 enzyme immunoassay, Fa. Syva, 58–136 nmol/l; T3 radioimmunoassay, Fa. Micro Medic, 1.5–3.5 nmol/l; iv TRH-test, 200 µg Proleulin, TSH-RIA, Fa. Henning, 2–25 mU/l) and presence of microsomal or thyroglobulin antibodies (Thy¬mune-M-HAT, < 1:1600; Thymune-T-HAT, < 1:40; Fa. Wellcome Reagents Ltd.).

Eight patients showed signs of endocrine orbitopathy stage III-IV according to the American Thyroid Association; thyroglobulin and microsomal antibodies were found in 6/20 and 14/20 patients, respectively. All patients had either signs of ophthalmopathy and/or elevated titres of microsomal and/or thyroglobulin antibodies.

Bioassay for thyroid stimulating antibodies (TSAb)

TSAb activity was evaluated by a modified in vitro bioassay according to Atkinson & Kendall-Taylor (1981), which determines the T3 release from thyroid slices. The method has been described in detail previously (Hörmann et al., in press). Briefly, thyroid tissue obtained at surgery and immediately transferred to an ice-cold buffer (modified Earle’s balanced salt solution with 20 mmol/l Heps, 5.6 mmol/l glucose, 1 g/l gelatine and 100 U/l insulin, pH 7.4) was cut into slices of a uniform size (0.5 × 1 × 1 mm) using a microtome and a punching machine. Two washed slices were placed in a bipartite pot of Teflon with a dialysis membrane (Fa. Kalle, Wiesbaden) that separated them from the lower compartment containing 5 ml of buffer. The slices were incubated in 0.1 ml of serum. The pots – 5 for each test sample – were incubated in a water bath at 37°C in an atmosphere of 95% O2 and 5% CO2 for 5 h. The slices were washed with buffer each when removed. After equilibrium dialysis (Ekins & Ellis 1975); (shaking water bath, 100 cycles/min, 30°C, 24 h), free T3 was determined in duplicate samples (0.3 ml) of the lower compartment by a sensitive RIA. Triplicate pots were simultaneously incubated in the absence of tissue to evaluate the dialysable free T3 of the individual serum. T3 released by the thyroid slices was calculated as difference of total free T3 and serum free T3.

Pooled Graves’ sera with high T3 releasing activity, bTSH (30 IU/mg, donated by Dr. Pierce, Los Angeles, USA) in a range from 0.1 to 10 IU/l, and pooled negative sera from 50 normals served as controls. Test samples had to release T3 exceeding the 2 SD-range of the negative controls to be regarded as positive (i.e. TSAb index > 30%).

Thyroid stimulating activity was expressed as TSAb index =

$$\frac{T_3 (\text{Graves’s serum}) - T_3 (\text{negative control})}{T_3 (\text{negative control})} \times 100\%$$

The intra-assay variation of the method at half maximal stimulation by 5 pooled Graves’ sera was 15% (n = 5) and the inter-assay variation 33% (n = 15).

Radioreceptor assay for TSH binding inhibiting immunoglobulins (TBII)

TBII were determined by a radioreceptor assay (TRAK-assay, Fa. Henning) using detergent solubilised
porcine TSH receptors according to Shewring & Smith (1982).

We have recently reported on methodological details and clinical application of this assay in a large number of patients (Hörmann et al. 1985). Briefly, test sera and controls were preincubated with the TSH receptors at 20°C for 15 min. The incubation with [125I]TSH was performed at 37°C for 60 min. Finally, receptors were precipitated by polyethyleneglycol and bound radioactivity was counted. TBII index = 

\[
(1 - \frac{\% \text{ bound } [125I] \text{TSH in Graves' serum}}{\% \text{ bound } [125I] \text{TSH in normal serum}}) \times 100\%
\]

TBII indices > 15% were defined as positive.

The intra-assay variability of a positive control (TBII index = 55%) was 4% (n = 5) and the inter-assay variability 12% (n = 10).

For comparative studies, all sera were tested for TSAb as well as TBII activities in a single assay to eliminate any influence of inter-assay variability.

Statistical methods
Statistical evaluations were made using Wilcoxon's test.

Results
Table 1 shows the basal and TSH (1.0 U/l) stimulated T₃ release in 8 goitrous thyroids, in tissue samples from 6 patients with Graves' disease under thiamazole and iodine treatment and in thyroids from 2 patients treated with propranolol only. The mean basal and TSH stimulable T₃ release in goitrous tissue was 17 ± 6 and 32 ± 12 pmol/l (x ± SD, n = 8). The maximum stimulation by TSH exceeded the basal T₃ release by 89 ± 32% (x ± SD, n = 8). In the 2 Graves' thyroids without antithyroid drug therapy (No. 1, 2) basal levels of T₃ released (68, 77 pmol/l) were higher than those in goitrous tissue (17 ± 6 pmol/l). The basal values from glands of 6 patients with antithyroid treatment (14 ± 3 pmol/l) did not significantly differ from those in goitrous tissue specimens. There was no stimulation by bTSH (range 0.1 to 10 U/l) in any Graves' tissue.

The T₃ releasing response in Graves' thyroid specimens was assessed for 20 sera from patients with active untreated Graves' disease. Seven sera were found to increase T₃ release in a Graves' gland from a patient with mild hyperthyroidism preoperatively treated with propranolol. The activity indices of the stimulating sera were 60 ± 21% (TSAb indices, x ± SD). Six of those 7 sera were also able to stimulate tissue specimens from patients pretreated with thiamazole or thiamazole and iodine with mean TSAb indices of 66 ± 17% and 69 ± 14%, respectively (Fig. 1). The stimulating effect of these 6 sera was confirmed in repeated experiments: each of them stimulated at least 4 different, bTSH resistant Graves' glands in vitro, irrespective of pretreatment. The seventh serum which had weakly stimulated the Graves' gland from a propranolol treated patient failed to activate 3 other Graves' thyroids (1 propranolol

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<td>Basal¹</td>
<td>23 ± 3</td>
<td>8 ± 2</td>
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<td>TSH²</td>
<td>48 ± 6</td>
<td>18 ± 3</td>
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<tr>
<td>Basal¹</td>
<td>68 ± 9</td>
<td>77 ± 11</td>
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<tr>
<td>TSH²</td>
<td>71 ± 11</td>
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Values (x ± SD, n = 5) are expressed in pmol/l T₃ released.
¹ T₃ release by pooled control sera of normals. ² T₃ release by 1.0 U/l bTSH.
* Preoperatively treated with propranolol. ** Preoperatively treated with thiamazole and iodine.
TSAb-activity (%)

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<th>goitrous tissue</th>
<th>Graves' tissue (methimazole+ iodine)</th>
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Fig. 1.
T\(_3\) releasing activity of Graves' sera \((n = 20)\) in goitrous tissue and Graves' thyroid pretreated with methimazole and iodine or propranolol. Incubated slices were prepared from one gland, respectively. Results are expressed as TSAb indices. TSAb index > 30\% was considered positive.

17/20 sera stimulated goitrous thyroid. The mean stimulating activity of these 17 sera was \(61 \pm 23\%\). All sera which stimulated Graves' slices were effective in goitrous tissue as well. The Graves' thyroid stimulating capacity of the individual serum, however, could not be predicted on the basis of its T\(_3\) releasing activity in goitre, since some of the highest stimulators in goitrous tissue failed to activate Graves' thyroid and a few weak stimulators were effective. On average, sera which stimulated Graves' slices revealed no higher stimulating activity in human goitrous tissue than serum samples which did not \((64 \pm 35\%, n = 6 \text{ vs } 59 \pm 17\%, n = 11; P = 9\text{s})\), excluding sera which were neither in goitrous nor in Graves' thyroid effective.

In addition, all sera were tested for TBII activity in a radioreceptor assay. The TBII indices of Graves' thyroid stimulating sera were significantly lower than the indices of the non-stimulating sera \((8 \pm 6\%, n = 6 \text{ vs } 40 \pm 17\%, n = 14, P < 0.01)\). To exclude the possibility that due to a bell-shaped

Dose response curves of 2 Graves' sera (○ and ●; appropriately diluted in pooled normal sera) in Graves' (left panel) and goitrous thyroid (right panel). Incubated thyroid slices had been prepared from one Graves' thyroid (pretreated with 50 mg/day methimazole and 100 mg/day iodine) and one goitrous gland. Results \((\bar{x} \pm s0, n = 5)\) are expressed as pmol/l released.
dose response curve TBII activity may be detectable only in a limited concentration range, 2 selected TBII negative, Graves' thyroid stimulating sera were assessed in a range of 0.1 to 50 µl of serum (appropriately diluted in pooled normal serum). There was no TBII activity detectable in the sera. In contrast, for 2 TBII positive sera a dose dependency could be established with a maximum activity (TBII indices 50 and 61%) in 40 to 50 µl and a still detectable activity in about 5 µl of serum.

No correlation between TBII indices and TSAb activities in goitrous tissue was found (r = -0.20, P = ns).

Discussion

The insensitivity of Graves' tissue to stimulation of cAMP accumulation by TSH as well as by Graves' immunoglobulins has been well documented in several studies (Kuzuya et al. 1980; Holmes et al. 1978). Our approach comparing the final response of hormone secretion in Graves' and human goitrous tissue confirms these data. Out of 20 sera from patients with active untreated Graves' disease 6 were found to act as stimulators of T₃ release in Graves' thyroid, whereas 17 could enhance T₃ release in goitrous tissue. These 6 sera in contrast to bTSH were effective in stimulating different Graves' glands in vitro, independent on preoperative treatment with propranolol, thiamazole or thiamazole and iodine. This argues against the hypothesis that pretreatment may be the critical determinant of Graves' tissue's unresponsiveness, although several authors (Onaya et al. 1978; Rapoport et al. 1976; Sherwin & Tong 1975; van Sande et al. 1975; Zakarija & McKenzie 1975) have well demonstrated an inhibitory effect of iodine and antithyroid drugs on cAMP response to TSH as well as thyroid stimulating immunoglobulins. Our observation of an increased basal T₃ release in Graves' thyroids in the absence of antithyroid drug therapy as well as data of Kasagi et al. (1980) of an increased basal adenylate cyclase activity indicate that glands have already been stimulated in vivo by endogenous thyroid stimulating antibodies. This prestimulation could lead to an insensitivity of the tissue via saturation or down-regulation of receptors. However, Graves' thyroid was not absolutely refractory to any further increase of hormone release in vitro, as some Graves' sera were able to stimulate T₃ release in Graves' slices.

The aim of this study was to characterize these sera which could overcome this insensitivity of Graves' tissue and to find out determinants responsible for their efficiency. One could suppose that the insensitivity of Graves' tissue may reduce the response to all Graves' sera by a similar degree in comparison to their activities in goitrous tissue. Accordingly, the most potent sera in goitrous tissue should have been expected to stimulate Graves' thyroid. Our results, however, were not consistent with this assumption. Actually, sera which stimulated Graves' slices revealed no higher stimulating activity in goitrous tissue than the other samples which did not, nor were some of the most potent stimulators in goitre effective in Graves' tissue. These findings clearly indicate that the height of antibody titre measured by T₃ release from goitrous tissue may not be the only determinant of the functional activation of Graves' thyroid in vitro. This is in good accordance with numerous reports (Teng et al. 1977; Docter et al. 1980) on a negligible correlation between anti-
body titre as evaluated by various techniques and the activity of Graves' disease in vivo.

In addition, the relationship between stimulating potency of Graves' sera in Graves' thyroid and height of hormone levels, incidence of microsomal or thyroglobulin antibodies, size of thyroids, stage of ophthalmopathy, age, sex of patients or additional diseases as well as treatment (data not shown) were analysed but no differences were observed in stimulating and non-stimulating sera. However, Graves' thyroid stimulating sera had a low or absent TBII activity as determined in a radioreceptor assay using solubilised porcine TSH receptors. From studies of Kohn et al. (1983) with monoclonal technique we know about the existence of immunoglobulins capable of stimulating adenylate cyclase but without inhibition of TSH binding. Their experiments led to the view that TSH receptor may be constituted by a ganglioside and glycoprotein component, as monoclonal antibodies which activated adenylate cyclase interacted with gangliosides, whereas monoclonals to the glycoprotein component rather blocked TSH function. Thus the final response of T₃ release probably depends on the interference of different autoantibodies characterized by different autoantigenetic sites, binding affinities (Carayon et al. 1983) and functional effects. This antibody spectrum is variably composed in the individual sera. Therefore, the effect of stimulating immunoglobulins may be modulated by the presence or absence of blocking antibodies in Graves' sera. Ginsberg et al. (1983) have recently demonstrated that in some sera cAMP stimulating activity was detectable at lower doses than receptor binding. Their explanation was that these sera acted as so-called 'TSH superagonists' stimulating in the presence of smaller amounts (undetectable by TSH receptor assay) of antibody bound.

Our results indicate that particularly these sera with a low or negligible TBII activity were potent activators of T₃ release from Graves' thyroid slices, whereas the stimulation of goitrous tissue could not be correlated to TBII activity. The accessibility and regulation of TSH receptors are likely to differ in human goitrous and Graves' tissue, which had been occupied and prestimulated in vivo by autologous thyroid stimulating immunoglobulins. De Bruin et al. (1984) demonstrated a decrease in the total number of TSH receptors but an increase in the number of high affinity binding sites induced by Graves' immuno-

globulins. Probably, in Graves' slices only a special population of biologically active antibodies with a high degree of TSH agonism i.e. high stimulating activity may induce a further stimulation. Overall, our findings shed new light on the stimulation of Graves' thyroid by Graves' sera in vitro indicating a crucial role of selected immunoglobulins with a high TSH agonistic potency. We conclude the composition of the antibody spectrum present in the sera, such as the occurrence of so-called 'superagonists', rather than the height of antibody titre as determined by various methods seems to be essential for their Graves' thyroid stimulating potency.

Acknowledgments

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References


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