Thyrotropin-binding inhibitory immunoglobulin in patients with Graves' disease

Measurement and relationship to numeric abnormality of T cells[1]

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Graves' autoantibodies were measured by thyrotropin-binding inhibitory immunoglobulin assay (TBII) in 19 patients with Graves' disease and in two patients with spontaneously resolving hyperthyroidism. T cell subsets in the peripheral blood were measured using monoclonal antibodies and flow cytometry (FACS II). The mean TBII index in patients with Graves' disease was 59.7 ± 2.8 and 68% had a positive TBII index. A higher sensitivity of TBII was observed in 13 patients with exophthalmos (77% positive) than in six patients with hyperthyroidism alone (50% positive). The mean ratio of T helper inducer cells (OKT-4 positive) to T suppressor cytotoxic cells (OKT-8 positive) in Graves' disease was 3.1 ± 2.0, which was significantly higher than controls (1.8 ± 0.4, $p < 0.01$). The increase in this ratio was primarily due to the decrease in the T suppressor cytotoxic cell population. Although there was a significant increase in mean ratio, eight patients had the ratio within normal range. Both patients with spontaneously resolving hyperthyroidism showed decreased T suppressor cell population but normal TBII levels. There was no significant correlation between T cell subsets and TBII in patients with Graves' disease (r = 0.33). The results suggest that patients with Graves' disease may have a numeric imbalance of immunoregulatory cells. However, this imbalance showed no significant relationship to the activity of the autoantibody.

Index terms: Goiter, exophthalmic · Immunoglobulins · Thyrotropin

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Graves' disease is an autoimmune disease characterized clinically by hyperthyroidism and diffuse enlargement of the thyroid gland with or without exophthalmos.[1] Hyperthyroidism in this disease is caused by circulating thyroid-

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Table 1. Characteristics of ophthalmopathy, associated thyroid disease, and treatment status in patients with Graves' disease

Ophthalmopathy	Thyroid Function	Status/Treatment
1. Severe prog.essive exophthalmos*	Hyperthyroid in past	Posttreatment, I-131
2. Severe exophthalmos*, bilateral diplopia	Hyperthyroid in past	Posttreatment, I-131
3. Severe exophthalmos, periorbital edema	Hyperthyroid in past	Posttreatment, PTU
4. Severe progressive exophthalmos*, periorbital edema, diplopia, upper lid retraction	Hyperthyroid (recurrent)	Posttreatment, PTU
5. Severe exophthalmos*, got worse posttreatment	Hyperthyroid (recurrent)	Posttreatment, subtotal thyroidectomy
6. Severe bilateral exophthalmos, periorbital edema, lid retraction	Hyperthyroid in past	Posttreatment, I-131
7. Bilateral malignant exophthalmos, diplopia, papilledema	Hyperthyroid in past	Posttreatment, I-131
8. Mild exophthalmos, tearing, blurred vision	Hyperthyroid in past	Posttreatment, I-131
9. Mild prominent eyes and stare	Hyperthyroid	Pretreatment
10. Mild exophthalmos bilateral (post-treatment)	Hyperthyroid	Posttreatment, I-131
11. Moderate exophthalmos, unilateral lid retraction	Euthyroid	Pretreatment
12. Severe unilateral exophthalmos*	Euthyroid	Pretreatment
13. Progressive pretibial myxedema	Hyperthyroid in past	Posttreatment, I-131
14. None	Hyperthyroid	Posttreatment, PTU
15. None	Hyperthyroid	Pretreatment
16. None	Hyperthyroid	Pretreatment
17. None	Hyperthyroid	Pretreatment
18. None	Hyperthyroid	Pretreatment
19. None	Hyperthyroid (recurrent)	Posttreatment, PTU

PTU = propylthiouracil.
* Progressive eye disease requiring surgical orbital decompression.

stimulating antibodies (TSI), which exert their action by binding to the thyrotropin (TSH) receptor protein present on the surface of thyrocytes.[2,3] Such autoantibodies have also been termed thyrotropin-binding inhibitory immunoglobulins (TBII) because they inhibit the binding of I-125-labeled TSH to thyroid TSH receptors in vitro.[3,4] Some evidence indicates that cell-mediated immunity is also involved in the pathogenesis of autoimmune thyroid diseases, including Graves' disease.[5–11] As in other autoimmune diseases, it has been postulated that a defect of suppressor T cell function might underlie the break in immunologic tolerance in patients with Graves' disease. However, results on quantitation of T cell subsets reported so far are conflicting.[12–16] Here we present our results on quantitation of T cell subsets in Graves' disease and our attempts to relate these results to the presence of TBII.

Methods

TSH binding inhibition assay

The TSH binding inhibition assay for the quantitation of autoantibody in Graves' disease was performed as previously described, with minor modifications.[3] Porcine thyroids were obtained from a local slaughterhouse, minced, and homogenized in 10-mM tris HCl buffer at pH 7.5. Following centrifugation at 500 × g and filtration, the crude membrane preparations were centrifuged at 15,000 × g for 20 minutes. The pellet was resuspended in 0.01-M tris HCl buffer at pH 7.5, 15-mM sodium chloride, and 0.1% BSA. The protein concentration of the membrane suspension was measured using the Lowry technique. Bovine TSH was obtained as a gift from Dr. John G. Pierce, and 2.5 μg of bovine TSH was labeled with 0.5 mCi (18.5 MBq) of $Na^{125}I$ using lactoperoxidase oxidation (BioRad Enzymobead Reagents). The labeled hormone was purified at 40°C by gel filtration (Sephadex G-100). Only the portion of I-125-labeled TSH that was capable of binding to thyroid membranes was used in the assay, i.e., the I-125-labeled bovine TSH was purified using thyroid membrane absorption (receptor purification) and the TSH bound to the membrane was eluted with sodium chloride and further purified on a gel filtration column (Sephadex G-100) prior to each assay.

For assay, crude porcine thyroid membranes

were incubated with patient or normal pooled immunoglobulin (1 mg) or excess TSH (for nonspecific binding) for 15 minutes at 37°C. After this incubation, receptor-purified bovine I-125-labeled TSH was added, and tubes were further incubated for one hour at 37°C. Then the tubes were centrifuged at 9,000 rpm in a microfuge, and the pellets were counted in a gamma counter. Nonspecific binding counts were subtracted from test sample counts, and the results were expressed as counts per minute (CPM) bound by the patient's IgG divided by CPM bound by the normal pool IgG.

Analysis of lymphocyte population by flow cytometry

Peripheral blood T helper inducer lymphocytes, T suppressor cytotoxic lymphocytes, and total T lymphocytes were quantitated using the Fluorescence Activated Cell Sorter (FACS II) (Becton Dickinson) and mouse monoclonal antibodies OKT-4 (T helper inducer), OKT-8 (T suppressor cytotoxic), and OKT-3 (total T lymphocytes) from Ortho Diagnostics. Briefly, after initial purification of mononuclear cells with Ficoll Hypaque, aliquots of the lymphocyte preparation ($1 \times 10^6/200\ \mu l$) were incubated with monoclonal antibody (5 μl) for 30 minutes at 0°C with frequent mixing. The cells were then washed twice with ice-cold medium and centrifuged at 300 × g for five minutes. Then the cells were stained with 100 μl of the appropriate dilution of fluorescein-labeled goat anti-mouse IgG (Cappel Laboratories) and were counted on the FACS II. As controls, lymphocytes were processed as described above but omitting the incubation step with monoclonal antibodies. The FACS was adjusted with glutaraldehyde-fixed chicken red blood cells, and appropriate gating to exclude undesired contaminating cells was carried out. At least 10,000 cells from each preparation were counted and the number of T helper (OKT-4) and T suppressor (OKT-8) cells was calculated by electronically dividing the 100× fluorescence profile by the total scattered profile of the test sample and subtracting a similar profile on the control sample. The helper-to-suppressor T cell ratio (OKT-4 to OKT-8 ratio) was calculated by dividing the percentage of OKT-4-positive mononuclear cells by the percentage of OKT-8-positive mononuclear cells.

Patients

A total of 43 samples was analyzed for T cell subsets. This included 22 normal healthy individuals and 19 patients with Graves' disease. Only 40 of these samples were analyzed for TBII, including 19 controls and 19 Graves' disease patients. The latter included 13 patients with exophthalmos (11 hyperthyroid and two euthyroid), one with pretibial myxedema, and six patients with hyperthyroidism without exophthalmos. The severity and characteristics of ophthalmopathy and the presence of hyperthyroidism, as well as status of the patient at the time of testing, are listed in *Table 1*. Also, out of these 19 patients with Graves' disease, nine were newly

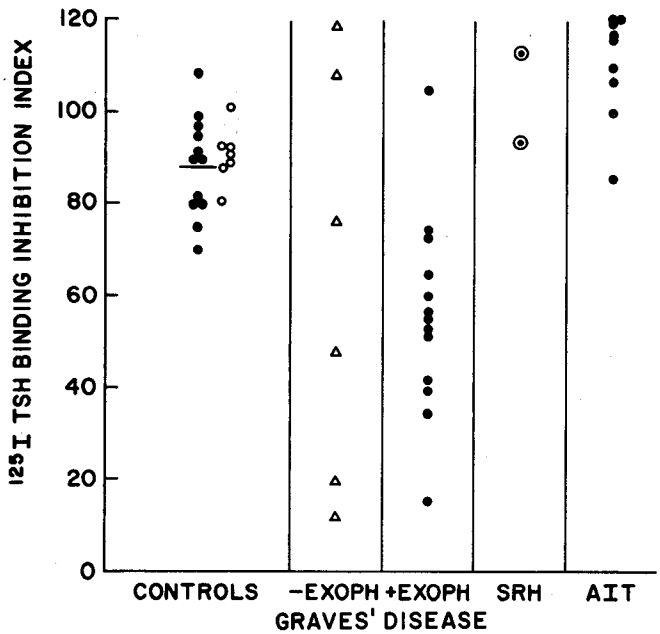

Fig. 1. Levels of TSH binding inhibitory immunoglobulins (TBII) in controls [healthy individuals (●) and patients with nonthyroidal illnesses (○)], in patients with Graves' disease [with (●) and without (△) exophthalmos], and in patients with spontaneously resolving hyperthyroidism (SRH) and autoimmune thyroiditis (AIT).

Table 2. TBII in controls and patients with Graves' disease

Group	Number	TBII Index	No. Positive
Controls	19	89.0 ± 10	—
Graves' disease (total)	19	57.0 ± 27*	13/19 (68%)
With exophthalmos	13	55.8 ± 21.8	10/13 (77%)
Without exophthalmos	6	61.0 ± 40.8	3/6 (50%)

TBII = thyrotropin-binding inhibitory immunoglobulin.
* $p < .0001$

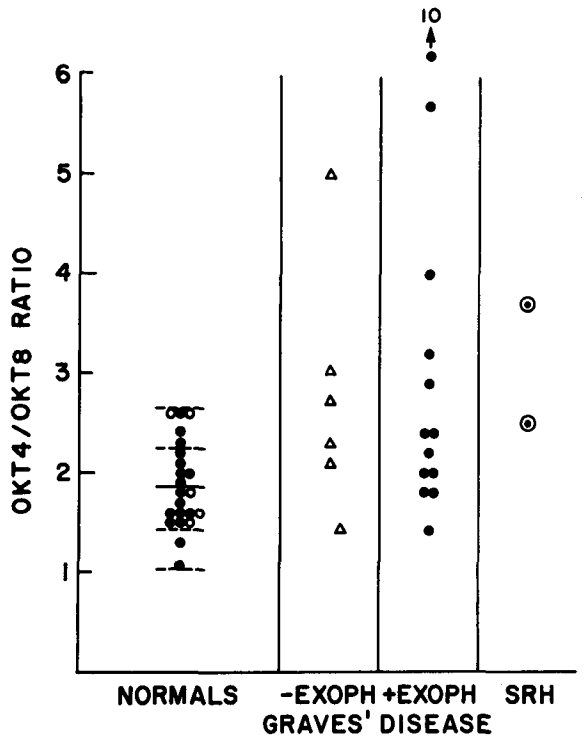

Fig. 2. Ratio of helper-inducer (OKT4) and suppressor-cytotoxic (OKT-8) T cells in normal controls, patients with Graves' disease [with (●) and without (△) exophthalmos], and patients with spontaneously resolving hyperthyroidism (SRH).

diagnosed untreated patients, seven had been treated in the past but had either recurrent thyroid disease or progression of eye disease, and the remaining three were considered to be stable post-treatment (one with propylthiouracil and two with I-131 treatment). In addition, two patients with spontaneously resolving hyperthyroidism (SRH) had low radioactive iodine thyroid uptake and were also studied.

The statistical significance of various measurements was assessed between controls and patients by use of the t-test at a probability level (p value) of 0.05. The correlation between TBII index and T4/T8 ratio was analyzed using linear regression and calculating correlation coefficient.

Results

Table 2 summarizes and *Figure 1* illustrates the actual results obtained for TBII in controls and in patients with Graves' disease. In normal healthy subjects, the mean TBII index was 89 ± 10 with a range of 69 to 109. Sixty-eight percent (13/19) of patients with Graves' disease were positive for TBII. Thirteen out of the 19 patients with Graves' disease had associated exophthalmos (one with pretibial myxedema) and 77% of these (10/13) were positive for TBII. Two were euthyroid with exophthalmos and one of these was positive for TBII. Six were hyperthyroid with no eye disease and three of these (50%) were positive for TBII. TBII was undetectable in two patients with SRH.

Table 3 summarizes the results obtained for total T cell and T cell subset quantitation by flow cytometry in these patients. The mean total T cells in normals was 77 ± 7.4% and in patients with Graves' disease was 66.9 ± 13.6%. The difference in total T cells between the two groups was statistically significant ($p < .01$). In Graves' disease the mean percentage of helper-inducer cells (OKT-4 positive) was 50 ± 13.7%, which was not significantly different from the mean percentage of helper-inducer cells found in controls (47.6 ± 7%). On the other hand, the percent of suppressor cells (OKT-8 positive) was only 19 ± 6.4% in Graves' disease, which was significantly lower than the normal group (normal, 25.6 ± 4.8%, $p < .001$). Also when we compared the T4/T8 ratio, the difference in the two groups was still statistically significant ($p < .01$) (*Fig. 2*). Although there was a significant increase in the T4/T8 ratio, eight patients with Graves' disease had ratios within 1 S.D. of controls (four were

Table 3. Peripheral T cell subsets in controls and patients with Graves' disease

	Total T cells (OKT3 +) (%)	Helper-Inducer (OKT4 +) (%)	Suppressor (OKT8 +) (%)	Ratio T4/8
Normal controls	77.0 ± 7.4	47.6 ± 7.0	25.6 ± 4.8	1.8 ± 0.4
Graves' disease (19)	66.9 ± 13.6*	50.0 ± 13.7†	19.0 ± 6.4	3.1 ± 2.0
Hyperthyroid and exophthalmos (13)	70.8 ± 8.9	53.0 ± 11.0	20.0 ± 6.8	3.2 ± 2.0
Hyperthyroid only (6)	59.6 ± 19.0	43.0 ± 17.0	16.0 ± 4.7	2.7 ± 1.2

* $p < .01$
† $p < .001$

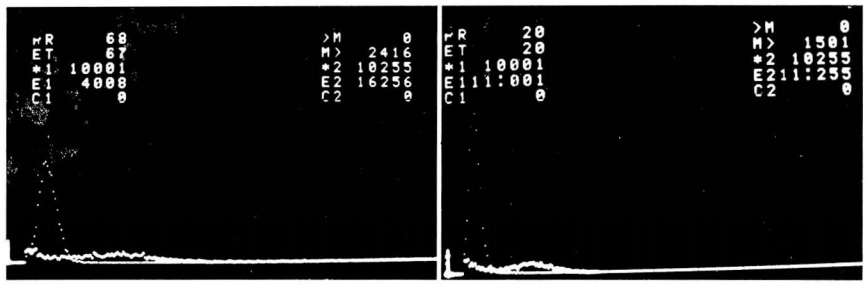

Fig. 3. Computer-overlapped histograms of the T4/T8 subsets of peripheral blood samples drawn from two patients with spontaneously resolving hyperthyroidism (SRH). The histograms show the decrease in T8 positive cell numbers but no change in the fluorescence intensity.

below the mean of controls). Interestingly, six out of these eight patients who had normal T helper-suppressor ratios were tested post-treatment. One of these six had recurrent disease (negative for TBII), three had progressive eye disease (two positive for TBII), and two were considered stable (posttreatment with propylthiouracil and I-131, both positive for TBII). In two patients with SRH, TBII was undetectable but the T4/T8 ratio was increased in one (3.7) and was high-normal (greater than 1 S.D.) in the other (2.5). These findings are illustrated in *Figure 3*. No statistically significant correlation was observed between TBII and T4/T8 ratios in these patients ($r = 0.33$).

Besides the quantitative difference observed in the number of T8-positive cells between Graves' disease and normals, we also found that the peak position of fluorescence intensity in the histogram showed a shift toward decreased fluorescence intensity in some of these patients. Most patients simply showed a decrease in number but no change in fluorescence intensity, with the exception of four patients who showed a remarkable decrease in fluorescence intensity of the T8 population (*Fig. 4*). Also, *Figure 5* shows a similar decrease in fluorescence intensity in a blood specimen obtained from a patient with Graves' disease before and one month after treatment with I-131.

Discussion

Volpé[17] in 1977 suggested that Graves' disease, like other autoimmune diseases, may be due to impaired immune regulation, possibly related to a defect in T suppressor lymphocyte function. Since then, a number of different methods for enumerating T cell subsets and for assessing the function of T suppressor lymphocytes have been applied to test this hypothesis. We analyzed total T cells and T cell subsets in patients with Graves' disease using monoclonal antibodies of OKT series and a Fluorescence Activated Cell Sorter (FACS II). Our results demonstrated a significant decrease in the percentage of total T cells (OKT-3 positive) and in suppressor-cytotoxic T cells (OKT-8 positive) but not in the number of helper-inducer (OKT-4 positive) cells. With respect to decrease in percentage of suppressor-cytotoxic T cells in Graves' disease, our data agree with findings of Thielemans et al[12] and with those of Sridama et al.[13] Both groups of investigators used the OKT series of monoclonal antibodies but a manual counting method. However, Iwatani et al,[16] using Leu series of monoclonal antibodies and flow cytometry, found a decrease in total T cells but no significant difference in the number of suppressor-cytotoxic T cells. Like Iwatani et al[16] we also observed a reduction in the peak position of fluorescence intensity in two hyperthyroid, one hypothyroid, and one euthyroid patient with Graves' disease. Our results also showed normalization of this shift after T4 replacement therapy in the hypothyroid patient and after I-131 treatment in one hyperthyroid patient. It is possible that this may reflect the degree of antigenic expression in these cells, which may be related to physiologic changes. The clinical significance of this shift in fluorescence intensity is not known at the present time. Our results further support the findings of Aoki et al,[18] Balázs et al,[19] and Hallengren and Forsgren,[20] who identified a defect in suppressor T cells using functional assays. We also found a persistence of the suppressor T cell abnormality in a number of patients after treatment.

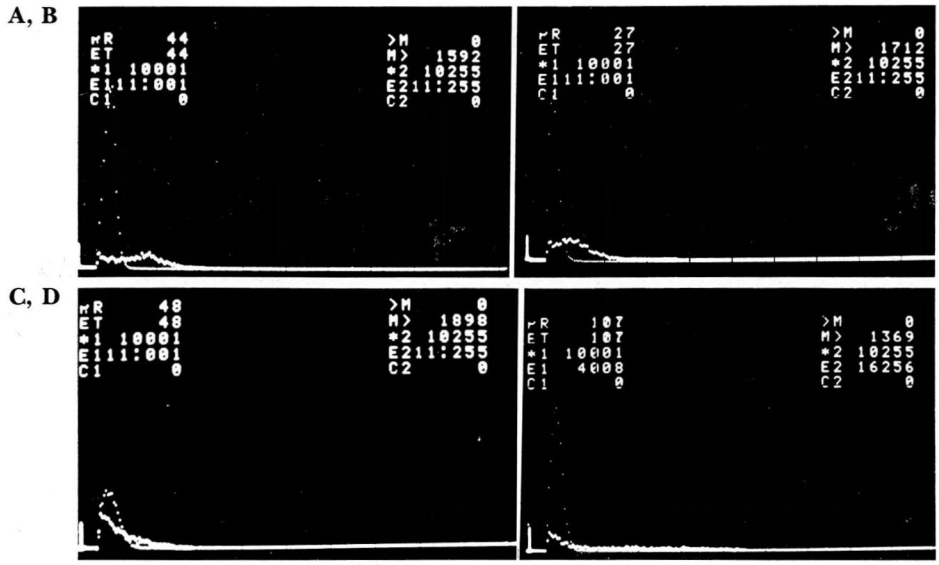

Fig. 4. Computer-overlapped histograms of the T4/T8 subsets in four patients, showing a remarkable decrease in fluorescence intensity of the T8 population without a substantial change in the T4 population.

A. Graves' disease treated in the past with I-131; severe exophthalmos, hypothyroid at the time of testing, showed normalization of pattern 1 month after T4 replacement.

B. Untreated euthyroid, unilateral exophthalmopathy.

C. Untreated hyperthyroid.

D. After I-131 treatment for Graves' disease, severe exophthalmopathy.

In addition to T cell subset quantitation, we also measured TSH receptor antibodies by measuring the inhibition of I-125-labeled TSH binding to TSH receptors. As this technique only detects the inhibitory activity and not necessarily the stimulating activity, the term TSH binding inhibition immunoglobulin (TBII) is more appropriate than thyroid stimulating antibody (TSI). TBII was detected in the majority of patients with Graves' disease in our study. The positivity was more impressive in patients who were hyperthyroid and had associated exophthalmos. We investigated possible relationship between the presence of autoantibodies and the numeric defect in T cell subsets and found no correlation between TBII positivity and decrease in T suppressor cells.

We also quantitated T cell subsets in two patients with painless thyrotoxic lymphocytic thyroiditis, also known as spontaneously resolving hyperthyroidism (SRH). Although the cause of this entity is unclear, these patients usually present with thyrotoxicosis, painless and small thyroid glands, low I-131 uptake, and biopsy evidence of lymphocytic thyroiditis.[21–24] Histopathologic studies have shown diffuse or focal lymphocytic infiltrate compatible with autoimmune thyroiditis.[21–25] We detected a significant decrease in T suppressor cells in both patients, with a significant increase in T4/T8 ratio in one patient and an increase greater than 1 S.D. above the normal range in the T4/T8 ratio of the other. In addition, a predominance of helper-inducer T cells was demonstrated immunomicroscopically in the thyroid lymphocytic infiltrates of one of these patients.[25] These findings suggest the immunologic nature of this disease. However, TBII was undetectable in both of these patients, a finding that is in agreement with previous reports.[22–25]

In conclusion, our results support the view that a numeric imbalance in immunoregulatory cells exists in patients with Graves' disease and that it is possible that this defect may be responsible for the initiation of the disease. However, this imbalance showed no relationship to the activity of Graves' autoantibody.

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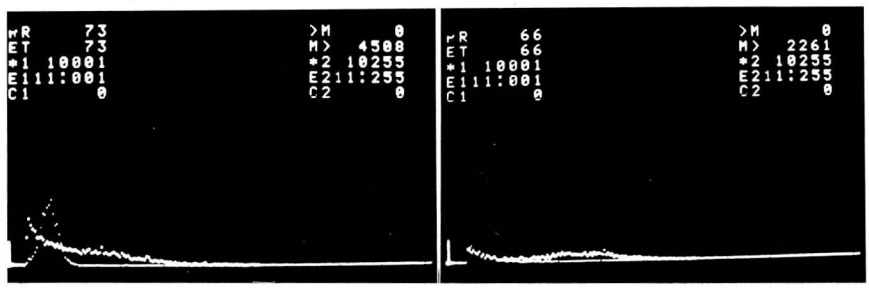

Fig. 5. Computer-overlapped histograms of the T4/T8 subsets from the same patient before (**A**) and two months after (**B**) I-131 treatment for Graves' disease. The histogram on the left (**A**) shows a remarkable decrease in fluorescence intensity of the T8 population without substantial change in the T4 population. The histogram on the right (**B**) shows the normalization of fluorescence intensity posttreatment.

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