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Research Article

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Antineutrophil Autoantibodies in Graves' Disease

Implications of Thyrotropin Binding to Neutrophils

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Abstract

The hyperthyroidism of Graves' disease may be caused by autoantibodies to thyrotropin (TSH) receptors. We have found that patients with this disease have autoantibodies to neutrophils as well, which can be displaced by TSH. Using a radiochemical opsonic assay, we found serum antibodies against homologous neutrophils in 6 of 11 Graves' patients. With a staphylococcal protein A-binding assay, we detected circulating antibodies to homologous neutrophils in 10 of 20 patients, while finding cell-bound antibody on autologous neutrophils in 7 of 8 (including 2 with negative serum tests). Use of human ^{125}I-TSH in a radioligand binding assay revealed that TSH bound to neutrophils rapidly (maximum binding within 10 min at 22°C, pH 7.4), specifically (<20% nonspecific binding), and reversibly. Adding TSH to the radiochemical assay resulted in a dose-dependent inhibition of opsonic antibody activity in serum from patients with Graves' disease. In contrast, TSH did not inhibit antibody activity of serum from patients with immune neutropenia not associated with thyroid disease.

Our findings suggest a basis for the association of Graves' disease with neutropenia. Furthermore, the discovery of such antineutrophil antibodies in Graves' disease permits detection of cell-bound antibody when free antibody is not present.

Introduction

The association of Graves' disease with neutropenia was first described by Kocher (1) in 1908, and subsequently confirmed (2). Other groups, studying patients with autoimmune neutropenia, have observed a number of individuals with concurrent or prior thyroid disease. The coexistence of both neutropenia and Graves' disease in some patients led us to study systematically a group of patients with Graves' disease for the presence of antineutrophil autoantibodies. Furthermore, we questioned whether the autoantibodies directed against thyroid thyrotropin (TSH)[1] receptors may cross-react with thyrotropin-binding moieties on neutrophils. To investigate this hypothesis, we studied the binding of thyrotropin to human neutrophils and the interactions in vitro of thyrotropin, antineutrophil autoantibodies, and neutrophils.

Methods

Patients. Sera and citrated whole blood were obtained from patients with Graves' disease and normal volunteers after informed consent. 22 patients were studied. There were 18 females and 4 males, with an age range of 23–74 yr. Their therapy and clinical status is presented in Table I. Patients were classified as having Graves' disease on the basis of the presence of diffuse goiter, exophthalmos, and a current or documented history of hyperthyroidism. Hyperthyroidism was defined on the basis of classical clinical symptoms, elevated serum thyroxine and triiodothyronine concentrations, and undetectable serum TSH levels. Patients were considered euthyroid by an absence of clinical symptoms of hyperthyroidism or hypothyroidism with normal serum thyroxine and triiodothyronine levels.

Neutrophils were purified by sedimentation of citrated blood with dextran (3). The leukocyte-rich supernatants were then centrifuged on Ficoll-sodium diatrizoate gradients, and erythrocyte contamination of the pellets was removed by hypotonic lysis.

IgG was purified by ammonium sulfate precipitation followed by ion exchange chromatography on diethylaminoethyl cellulose (4).

Antineutrophil antibodies were assessed by two methods. The cell:cell recognition assay was used to detect antineutrophil antibody opsonic activity in serum as previously described (3, 5). This assay is based on the observation that normal neutrophils recognize other neutrophils opsonized with antibody and respond with an increase in glucose oxidation. 2.4×10^6 normal neutrophils were suspended in 0.2 ml of Krebs-Ringer-phosphate medium (KRP), pH 7.4, and incubated (sensitized) for 30 min at 22°C in 0.4 ml test serum. After being washed, these cells (designated "test cells") were resuspended in 0.2 ml KRP. An equal number of normal neutrophils (designated "indicator cells") was not exposed to serum. Instead, they were incubated for 60 min in 0.8 ml KRP containing 100,000 cpm of [1-^{14}C]glucose. Indicator and washed test cells were then brought together in a total volume of 1 ml KRP in sealed polypropylene vials with a strip of hyamine-impregnated filter paper inserted through the top. The amount of radioactive CO_2 evolved after 30-min incubation at 37°C was determined by counting the filter paper in a scintillation spectrometer. The mean of control incubations (test cells sensitized with normal IgG or serum) was defined as 100% of control activity. In some experiments, varying amounts of bovine TSH (bTSH, Thytropar; Armour Pharmaceutical Co., Tarrytown, NY) were added to test cell suspensions before adding serum.

A second method, the staphylococcal slide assay (6), enabled us to look for IgG antineutrophil antibodies directly bound to autologous neutrophils as well as for free IgG in the serum capable of binding to homologous neutrophils. Dextran-sedimented leukocyte suspensions (autologous or homologous) were allowed to adhere to glass slides, fixed in paraformaldehyde, and washed. The slides were then covered with 50 μl of test or control serum and incubated for 30 min at 22°C. Excess serum was then rinsed off with calcium-free KRP buffer, pH 7.4, containing 40 mg/ml bovine serum albumin (Sigma Chemical Co., St. Louis, MO). In each assay, two known positive and two known negative control sera were evaluated in triplicate in parallel with the test sera. In a direct assay, this serum sensitization step was omitted. After sensitization, 50 μl of protein A containing *Staphylococcus*

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1. *Abbreviations used in this paper:* bTSH, bovine thyrotropin; hTSH, human thyrotropin; KRP, Krebs-Ringer-phosphate medium; TSH, thyrotropin; TSI, thyroid-stimulating immunoglobulins.

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Table I. Summary of Patients and Antibody Test Results

Patient no.	Age	Sex	Treatment*	Clinical status‡	Total leukocyte count	Total neutrophil count	Antineutrophil antibodies: Indirect test (homologous cells): Opsonic test	Antineutrophil antibodies: Indirect test (homologous cells): Slide test	Antineutrophil antibodies: Direct test (autologous cells): Slide test
					mm³	*mm³*			
1	36	F	I^{131}	Euthyroid	—	—	Positive	Positive	—
2	23	F	I^{131}	Hyperthyroid	5,100	1,300	—	Positive	—
3	36	F	Propylthiouracil	Euthyroid	2,650	100‖	Negative	Positive	—
4	30	F	Methimazole	Euthyroid	7,700	—	Positive	Negative	—
5	37	F	I^{131}	Euthyroid	4,200	2,300	—	Negative	—
6	53	F	I^{131}	Hypothyroid	6,600	3,600	Positive	Positive	—
7	50	M	I^{131}	Euthyroid	7,700	5,200	Positive	Positive	—
8§	49	M	None	Euthyroid	10,600	5,800	Positive	Positive	—
9	37	F	I^{131}	Euthyroid	6,800	—	Negative	Negative	—
10	68	F	I^{131}	Euthyroid	6,500	4,000	Negative	—	—
11	43	M	I^{131}	Euthyroid	—	—	Negative	Negative	—
12	26	F	None	Hyperthyroid	2,900	1,400	—	Negative	Positive
13	34	F	I^{131} Propylthiouracil	Euthyroid	8,900	4,900	—	Negative	—
14	74	F	I^{131}	Hyperthyroid	8,400	6,700	—	Positive	Positive
15	34	F	I^{131}	Euthyroid	4,600	2,800	—	Positive	Positive
16§	57	F	None	Euthyroid	—	—	—	Positive	Positive
17	36	F	I^{131}	Euthyroid	5,500	4,900	—	—	Positive
18	20	F	Methimazole	Hyperthyroid	800	30‖	Negative	Negative	Positive
19	32	F	None	Hyperthyroid	3,000	1,900	—	Negative	Negative
20	53	M	None	Hyperthyroid	6,400	4,000	—	Negative	—
21	36	F	None	Hyperthyroid	4,600	2,200	Positive	Positive	Positive
22	33	F	None	Hyperthyroid	6,000	3,400	—	Negative	—

* Treatment: I^{131}, therapeutic radioiodine at any previous time. Propylthiouracil or methimazole, under treatment with these agents at time of study. ‡ At time of study. § Euthyroid (ophthalmic) Graves' disease. ‖ Patients with acute granulocytopenia thought to be secondary to antithyroid drugs.

aureus Cowan 1 suspensions was layered onto the cells and incubated for 30 min at room temperature. These organisms, cultured as previously described (7), had been killed by incubation in 0.5% formaldehyde for 3 h at 20°C, and heat-treated at 80°C for 3 min. Bacteria were prepared by suspending 100 ml of the stock in 2 ml of suspension solution (0.05% Triton X-100, 0.15 mM NaCl, 5 mM Na_2 EDTA, 0.2 mg/ml sodium azide, pH 7.4), and centrifuged at 12,000 *g* for 30 s. To the resultant pellet of 50 μl of packed staphylococci, 1 ml of suspension solution containing 200 mg/ml bovine serum albumin was added and swirled with a vortex mixer to form a suspension. Slides were then immersed sequentially in bovine albumin solutions of decreasing concentrations in KRP buffer. The slides were then stained, inspected under light microscopy, and the percentage of neutrophils that bound three or more staphylococci was determined. Each test was done in triplicate, at least 100 cells were observed, and the average percentage of labeled neutrophils was recorded. A test was considered positive for antineutrophil antibodies if >20% of neutrophils was labeled. Validation of criteria for positive assays has been described (5). The rate of false positive tests is <4%.

Hormone preparation. Highly purified human thyrotropin (hTSH) was provided by the National Pituitary Agency, Baltimore, MD, with an immunopotency of 3–5 U/mg of Mill Hill Reference Standard B. This material was iodinated with ^{125}I by the lactoperoxidase method (8) to a specific activity of 70–100 μCi/μg and then separated from free iodine by filtration over a 90 × 1.5 cm Sephadex G100 chromatographic column. This material was 85–100% immunoprecipitable after incubation with excess rabbit anti-hTSH serum at final dilution of 1:1,000 (9).

Highly purified bTSH was generously supplied by Dr. John Pierce. Cruder preparations of bTSH were obtained from Armour Pharmaceutical Co. Purified human luteinizing hormone (LER-960) and follicle-stimulating hormone (NIH-FSH-HS-1) were obtained from the National Pituitary Agency.

hTSH binding to neutrophils was assessed by a radioligand binding assay. 5×10^5 gradient-purified neutrophils were incubated with varying amounts of ^{125}I-hTSH and test material in isotonic KRP buffer, pH 7.4, with total incubation volumes of 700 μl. To terminate incubations, 500-μl aliquots of the suspensions were removed and placed immediately into plastic tubes containing 3 ml of iced buffer. These tubes were then immediately inverted over glass fiber filters (GF/A, Whatman Laboratory Products, Inc., Clifton, NJ) that had been mounted in a vacuum suction manifold and presoaked with excess TSH (>10^{-5} M). This presoaking resulted in negligible counts being bound to the filters in the absence of cells. Under vacuum suction, suspension media were removed and the cells were washed with 12 ml of iced buffer. The filters were then counted for ^{125}I in an autogamma spectrometer. "Specific binding" was defined as the amount of the total bound radioactivity that was displaced by an excess of unlabeled bTSH (>10^{-5} M) that had been added to the incubations concurrently with the radiolabeled TSH.

To assess whether deiodination or degradation of hormone occurred, 5×10^5 neutrophils were incubated with ^{125}I-hTSH for various times

at both 22° and 37°C. At the completion of incubation, the cells were pelleted by centrifugation at 12,000 *g* for 1 min, and the supernatant was assayed for immunoprecipitability of radioactivity, using rabbit anti-hTSH serum as noted above. This was compared with immunoprecipitability of preincubation samples of labeled hTSH.

Reversibility of binding was investigated by incubating cells with ^{125}I-hTSH as described above, subsequently adding excess unlabeled hormone to the incubation, and then determining residual counts bound to cells.

Results

Antineutrophil antibodies. Using the opsonic method to assay serum against homologous neutrophils, 6 of 11 Graves' patients had antibodies. Using the staphylococcal assay for the same purpose, the sera of 10 of 20 patients were positive. When we studied the autologous neutrophils of eight patients by the staphylococcal slide test, seven of them were found to have cell-bound antibodies, including two in whom the sera were negative for binding of antineutrophil IgG to homologous cells. Antibodies to lymphocytes were not found. As shown in Table I, antibodies were detected even in patients with normal neutrophil counts. Neutropenia (<1,800/mm^3) was observed in two patients off treatment as well as in two patients who were possibly neutropenic because of reaction to propylthiouracil or methimazole.

Inhibition of antineutrophil opsonic antibody activity by TSH. When bTSH was added to test cell incubations, the opsonic activity of these antibodies was inhibited in a dose-dependent fashion (Fig. 1). bTSH did not, however, inhibit the opsonizing activity of serum from two patients with immune neutropenia who had no history of, or evidence of, thyroid disease (Fig. 1).

Binding of hTSH to neutrophils. hTSH was noted to bind rapidly, stably, and reversibly (Fig. 2) to human neutrophils. Specific binding (as defined above) varied between 2.5 and 16% of the added counts. Nonspecific binding was always <20% of the specific binding and generally much lower (in many experiments, there was no nonspecific binding in excess of the cell-free control filter blanks). The effects of pH on this system are also illustrated in Fig. 2. As noted by others (10, 11), specific binding to TSH is greatest at low pH, intermediate in the physiologic range, and lowest at alkaline pH. Effects of temperature are shown in Table II. While there was little difference in binding at 22° and 37°C, parallel incubations at 0°C resulted in smaller amounts of hormone bound. Reversibility of binding was demonstrated by incubating cells with ^{125}I-hTSH for 15 min, and then adding excess (>10^{-5}) bTSH. Within 5 min, all labeled TSH was displaced from the neutrophils (Fig. 2, top, dashed line). The displaced ^{125}I-hTSH was 85–95% immunoprecipitable (the same as control ^{125}I-hTSH), suggesting that degradation or deiodination did not occur during the incubation.

To investigate further whether the interaction of TSH with neutrophils suggested characteristics of a physiologic receptor, we subjected the radioligand binding data to Scatchard analysis (12, 13). Under conditions used in these assays, we were not

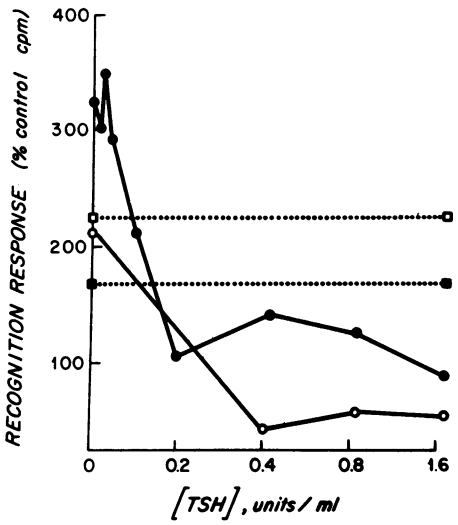

Figure 1. Effects of TSH on opsonic activity of antineutrophil antibodies. Test cells were exposed to antisera in the presence of varying amounts of crude bTSH for 30 min at 22°C, washed, and incubated with indicator cells for 30 min. Ordinate shows recognition response ([1-^{14}C]glucose oxidation to $^{14}CO_2$ by indicator cells) as percentage of control (recognition response in presence of cells sensitized with normal serum). Solid lines, serum from two different patients with Graves' disease. Dashed lines, serum from patients with autoimmune neutropenia (lower) and drug-induced immune neutropenia (upper). Each point represents mean of 2 or 4 determinations. ● and ○, Graves'. ■ and □, other immune neutropenia.

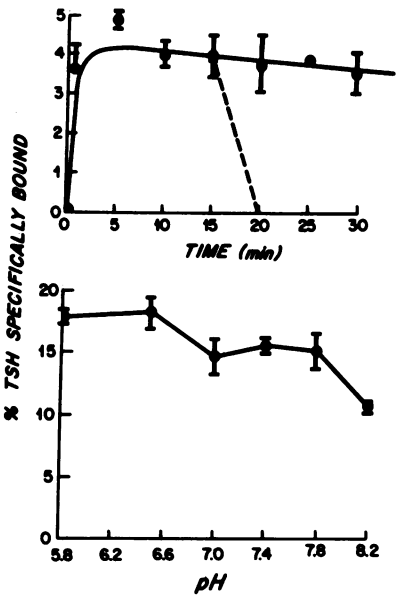

Figure 2. Upper graph: time course of specific hTSH binding to neutrophils. 9,400 cpm human ^{125}I-TSH was added to 5 × 10^5 neutrophils at 22°C, pH 7.4, and incubated for the times indicated. Parallel incubations with excess unlabeled bTSH (10^5 M) were performed to define nonspecific binding. Ordinate shows TSH specifically bound (total binding minus nonspecific binding) as percent of total labeled TSH added. Dashed line, cells incubated with labeled hormone for 15 min, then excess unlabeled TSH added. Each point represents mean and range of two experiments, each with duplicate determinations. Lower graph: effects of pH on TSH binding. 19,000 cpm ^{125}I-TSH was added to 5 × 10^5 neutrophils at 22°C for 15 min at the indicated pH. Specific binding was determined as above. Each point represents mean and range of duplicate determinations.

Table II. Effect of Temperature on Specific Binding of Human ^{125}I TSH to Neutrophils

Temperature	% TSH specifically bound	
	10-min incubation	20-min incubation
0°	10.1±0.9	10.8±2.3
22°	14.6±2.6	15.3±2.7
37°	15.7±2.4	14.6±1.7

19,000 cpm of ^{125}I-TSH were added to 5×10^5 neutrophils, pH 7.4, and incubated for the times indicated. Results are expressed as mean±SE (4–6 determinations) percent specific binding.

able to demonstrate consistently the high affinity binding characteristic of peptide hormone receptors.

Additional information about the specificity of human TSH binding to neutrophils is shown in Fig. 3. Unlabeled human TSH was much more efficient at competing with labeled human TSH for binding to neutrophils than was unlabeled purified bTSH. Neither follicle-stimulating hormone nor luteinizing hormone had competing activity.

Discussion

The major points in this study are (*a*) patients with Graves' disease frequently have antineutrophil autoantibodies, (*b*) TSH inhibits the opsonic activity of these antibodies, and (*c*) TSH binds to neutrophils rapidly and reversibly.

TSH binding has been described with a variety of tissues (10, 11, 14–16). Published data on the binding characteristics vary widely, depending on both the methods used and the species source of TSH and cell (or membrane) preparations. Reports from other laboratories agree with our observations of certain important features, such as the rapid time course of binding (14) and pH dependence (10, 11). Also, receptors for porcine TSH have been described on human neutrophils (16). Whether these neutrophil binding sites represent biologically important "physiologic receptors" is not certain, but some studies show that TSH may initiate neutrophil cyclic AMP production and may be relevant in neutrophil function (17).

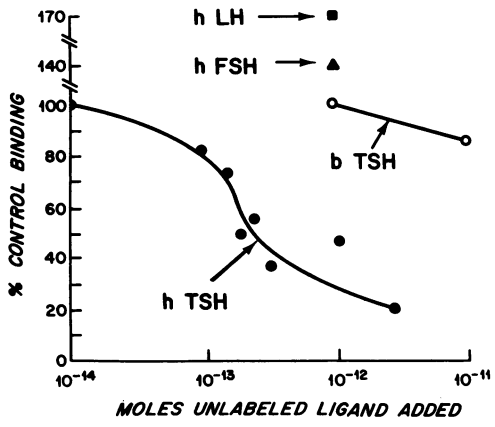

Figure 3. Specificity of TSH binding. Neutrophils were incubated at 22°C, pH 7.4, for 15 min with human ^{125}I-TSH and unlabeled ligands in the amounts indicated. Results are expressed as percent of control specific binding (specific binding in the absence of unlabeled ligands). Each point represents mean of duplicate determinations.

Hyperthyroidism in Graves' disease is thought to be due to autoantibodies directed against TSH receptors on thyroid cells (18–24). In most cases, immunoglobulins that inhibit binding of radiolabeled TSH to thyroid receptors (thyrotropin binding inhibiting immunoglobulins) are demonstrable in sera from these patients. Other assays, which do not necessarily correlate with the radioligand binding methods, demonstrate that the immunoglobulins from these patients behave as TSH agonists, i.e., activate adenylate cyclase or stimulate colloid droplet formation in thyroid preparations (thyroid-stimulating immunoglobulins or TSI). Recent studies with monoclonal antibodies argue that the antibodies that inhibit TSH binding to thyroid membranes are not the same as those that stimulate the gland (21, 22). Since the antineutrophil antibodies described here behave in a manner similar to the thyroid thyrotropin displacing immunoglobulins, it is possible that these two types of autoantibodies are actually the same, i.e., directed against the same or similar antigens expressed on more than one tissue.

The finding of circulating antineutrophil antibodies in 50–55% of our patients raises several questions, including (*a*) Why were so few of these individuals neutropenic? and (*b*) If these antibodies are related to those presumed pathogenic in Graves' disease, why were they not found in a higher fraction of patients? At least two possible explanations exist for the first question. First, it is possible that some of the antibodies may not, in vivo, alter the survival of the neutrophils against which they are directed, or, second, in cases where cell lifespan is shortened, increased marrow production might compensate (similar to the compensated hemolytic state of some erythrocyte disorders). The second question is more complex, but some of our data may help approach its answer. While serum antibodies against homologous cells were not found in almost half the patients, cell-bound IgG antibody was observed on autologous cells of seven of eight patients so studied. These findings are similar to the results of Coombs' antiglobulin test in autoimmune hemolytic anemia. While cell-bound antibody may be found in almost all cases (i.e., the direct antiglobulin test), the indirect test for serum antibody can be negative from 20 to 65% of the time, depending on the method of erythrocyte preparation (25). This analogy might also explain some of the false negative tests for TSI or thyrotropin displacing activity that can be observed in Graves' patients (26). Until recently (21, 22), these negative results occurred often enough to have cast doubt on the model of Graves' disease as an autoimmune process with antibodies directed against TSH receptors. However, it seems reasonable to postulate, on the basis of what is known about autoimmune hemolytic anemia and the neutrophil data shown here, that in some cases of Graves' disease, most all of the TSI or thyrotropin displacing activity could be cell-bound (either on thyroid or other cells), and that serum levels might be below limits of detection.

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