# Autoantibodies to Insulin Are Present in Sera of Patients With Autoimmune Thyroid Disease

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**It has been clinically suspected that patients with autoimmune thyroid disease are at an increased risk of developing other autoimmune diseases later in life. To determine the presence and potential importance of a more generalized deregulation of immune response in patients with Graves' disease and Hashimoto's disease, sera from 33 patients with Graves' disease and 16 patients with Hashimoto's disease were screened for the presence of anti-insulin antibodies and anti-insulin-receptor antibodies. An enzyme-linked immunosorbent assay was used to identify the presence of IgG against human insulin. The optical density indicating the presence of IgG against insulin in sera from patients with Graves' disease averaged .172 ± .024 (mean ± SE; range .010–.802), compared to the mean normal value of .098 ± .0009 (range .012–.238) in 33 control subjects. Ten of 33 patients with Graves' disease had values >.200, whereas control sera values were <.200 in all but one case ($P < .005$, Graves' sera vs. controls). The sera from patients with Hashimoto's disease had a mean optical density of .110 ± .016, with 15 of 16 values between .010 and .200. These values were not significantly different from controls with an insulin-binding inhibition assay. Anti-insulin-receptor antibodies were not detected in any of 33 patients with Graves' disease, and cytoplasmic islet cell antibodies were not detected in sera from seven patients with Graves' disease who had insulin-binding antibodies. These data support the hypothesis that the immunologic response in autoimmune thyroid disease may be more heterogeneous and polyclonal than previously believed. *Diabetes* 37:317–20, 1988**

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Graves' disease and Hashimoto's disease are autoimmune disorders characterized by the presence of circulating anti-thyroid antibodies (1). In Graves' disease, autoantibodies are formed against the thyroid-stimulating hormone (TSH) receptor, whereas in Hashimoto's disease, autoantibodies are formed against thyroglobulin and microsomal components. Although the mechanisms by which thyroidal antibodies are formed remain uncertain, data are accumulating to indicate that antigen-specific suppressor T-lymphocyte defects may be involved in this process. Topliss et al. (2) have shown that peripheral T-lymphocytes from patients with Graves' disease exhibit decreased lymphokine production when cultured in the presence of thyroid antigens, whereas peripheral T-lymphocytes from insulin-requiring diabetic subjects could correct this abnormality. In contrast to these data, which support a specific T-lymphocyte defect, Weetman et al. (3) have demonstrated abnormal stages of B-lymphocyte activity in the circulation of patients with autoimmune thyroid disease. In addition to possible abnormalities in T- and B-lymphocyte function, autoantigens such as thyroglobulin added in vitro may be capable of stimulating antibody production in lymphocytes of autoimmune disease patients (4–6). Patients with autoimmune thyroid diseases may also be predisposed to develop other autoimmune disorders such as diabetes mellitus, myasthenia gravis, and collagen vascular diseases (7). To further examine the heterogeneity of the autoantibody production in patients with autoimmune thyroid disorders, we examined sera from patients with Graves' disease and Hashimoto's disease for both insulin antibodies and insulin-receptor antibodies.

## MATERIALS AND METHODS

**Reagents.** Human insulin was purchased from Lilly (Indianapolis, IN), microtiter plates (Immunlon 2) were purchased from Dynatech (Alexandria, VA), and affinity-purified alkaline phosphatase–conjugated anti-human IgG (Fc specific) anti-

serum and *p*-nitrophenyl phosphate were purchased from Sigma (St. Louis, MO). Tween 20 was purchased from Fisher (Fairlawn, NJ), and $^{125}$I-labeled insulin was purchased from New England Nuclear (Boston, MA).

**Patients.** Sera from 33 patients with Graves' disease, 16 patients with Hashimoto's disease, and 33 normal subjects were obtained after informed consent. The diagnosis of Graves' disease was based on an elevated $T_4$ and thyroidal $^{131}$I uptake, in the clinical context of thyrotoxicosis and goiter. The diagnosis of Hashimoto's disease was based on a high titer of anti-microsomal or anti-thyroglobulin antibodies and clinical thyroiditis or hypothyroidism. Thyroid-stimulating immunoglobulins (TSI) were measured in two patients with Graves' disease by an in vitro bioassay with a thyroid cell line (FRTL-5; Nichols, San Juan Capistrano, CA); the results were negative in one patient and positive in the other. TSH-receptor–binding inhibitory immunoglobulins (TBII) were measured in 15 patients with Graves' disease and in 2 patients with Hashimoto's disease with solubilized TSH receptor from porcine thyroid cell membranes (Nichols) and were positive in 11 patients with Graves' disease and 1 of the patients with Hashimoto's disease. Most of the patients with Graves' disease were being treated with antithyroid drugs (usually propylthiouracil) when serum was collected. There was no attempt to correlate therapy with presence of antibody; however, several of the patients with autoimmune thyroid disease had evidence of other immune phenomena, including 5 patients with ophthalmopathy (4 Graves' disease, 1 Hashimoto's disease), 4 patients with systemic lupus erythematosus or rheumatoid arthritis (1 Graves' disease, 3 Hashimoto's disease), and 1 patient who had both Graves' disease and immune thrombocytopenic purpura. Several patients with type I (insulin-dependent) diabetes mellitus and either Graves' disease or Hashimoto's disease were excluded from this study. No patient was known to have received insulin injections. All patients had normal random blood glucose values on at least one occasion. A review of the patients' charts failed to reveal the diagnosis of diabetes or elevated blood glucose values. The family history of diabetes or other autoimmune disease is unknown.

**Enzyme-linked immunosorbent assay (ELISA).** Microtiter plates were coated with human insulin in a concentration of 1 μg/ml and incubated at 4°C until used. Assays were run within 5 days of preparing plates (8). The plates were then washed with 10 mM phosphate and 150 mM NaCl buffer (pH 7.2; PBS), and each well was blocked for 1 h at 32° with PBS–1% bovine serum albumin (BSA). The plates were washed five times with PBS containing 0.05% Tween 20 and 0.1% BSA. Serum samples to be tested were diluted 1:10 with PBS (pH 7.2), and 50 μl of sera was placed in duplicate wells. After incubation for 1 h at 37°, plates were washed with PBS-Tween–0.1% BSA, 50 μl of a 1:1000 dilution of affinity-purified alkaline phosphatase–labeled anti-human IgG was added to each well, and wells were incubated for 1 h and washed five times with PBS-Tween–0.1% BSA. Finally, 100 μl of *p*-nitrophenyl phosphate in diethanolamine buffer (pH 9.6) was added to the duplicate wells, wells were incubated in the dark, and the color change was read when positive control samples reached an optical density of 1.000 at 405 nm. For purposes of intra- and interassay comparisons, one negative and two positive control serum samples in several dilutions were included on each plate. Data were included for analysis when there was ≤10% variability in optical density between duplicate samples. Each sample reported here gave similar optical density measurements, with <10% variation when repeated on a different plate.

**Binding inhibition insulin-receptor–antibody assay.** Human lymphoblastoid cells (IM-9) were grown to stationary phase at 37° in RPMI-1640 and supplemented with 3% glutamate and 10% fetal calf serum in plastic flasks (9). Viability of cells was determined to be >90%. IM-9 cells were sedimented and resuspended at a concentration of $10 \times 10^6$ in a lymphocyte assay buffer containing 50 mM HEPES, 120 mM NaCl, 2.5 mM KCl, 1 mm EDTA, 15 mM sodium acetate, 10 mM glucose, and 10 mg/ml BSA (insulin free) at pH 7.6. A 0.4-ml cell suspension was incubated at room temperature with 0.1 ml of a 1:2 dilution of test serum and PBS (pH 7.2). After 1 h, cells were sedimented and washed twice with 2 ml of lymphocyte buffer. The pellet was then resuspended in 0.5 ml of lymphocyte assay buffer containing 100 pg $^{125}$I-insulin and incubated at 12°C for 2 h (8). Duplicate samples were prepared, with one set containing unlabeled insulin in a concentration of 10 μg/ml. After incubation, 0.2 ml was aliquoted in duplicate into microfuge tubes (PGC Scientifics, Gaithersburg, MD) containing 0.15 ml lymphocyte buffer and centrifuged for 45 s. The supernatant was aspirated and discarded, the microfuge tips containing the pellet were cut, and the radioactivity of the pellet was determined. Specific $^{125}$I binding was determined as the difference between tracer binding in the absence and presence of unlabeled insulin. Cytoplasmic islet cell antibodies were determined by the method of Srikanta et al. (10).

**Statistics.** Statistical tests used were $\chi^2$-analysis and Mann-Whitney-Wilcoxon tests. Statistical calculations were performed by a computer program purchased from Statgraphics/STSC (Rockville, MD). All values are expressed as means ± SE unless otherwise noted.

## RESULTS

**Insulin binding of IgG from normal sera.** Sera from 33 normal subjects had a mean optical density at 405 nm of .098 ± .009 (SD .05, range .032–.238; Fig. 1). Only 1 of the 33 samples had an optical density >.2. Test values >2SD above the control mean (i.e., >.200) were considered abnormal.

**Insulin binding of IgG from autoimmune thyroid disease sera to insulin.** Binding of sera from 33 patients with Graves' disease to wells coated with human insulin resulted in a mean (±SE) optical density of .172 ± .024 (range .034–.802; Fig. 1). Ten of 33 patients had values >.2. Four (12%) of 33 patients studied had optical density values >.248, which is 3SD above the mean normal value, and it is also above all normal serum optical density values. The mean binding in Graves' disease patients' sera was significantly higher than normal sera ($P < .005$). Binding was not correlated to severity of illness, presence of ophthalmopathy, type or duration of therapy, or serum levels of TSI and TBII. All of the patients tested had normal random blood glucose levels on at least one occasion. Although the patients included in this study generally had a high incidence of other autoimmune disease as noted in MATERIALS AND METHODS, none of the

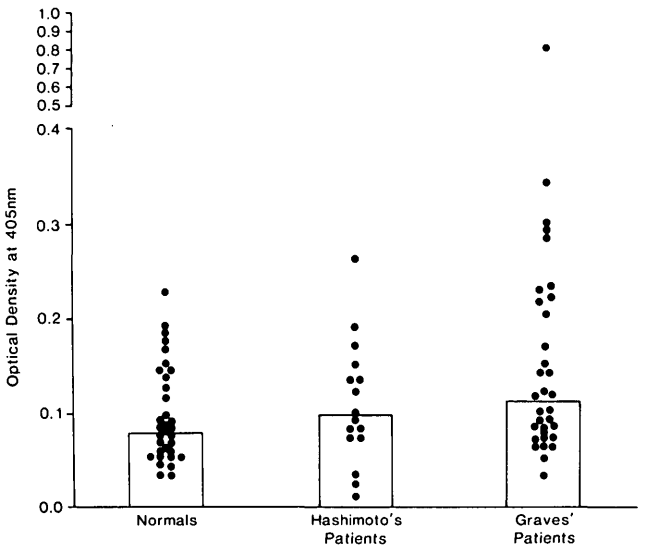


**FIG. 1. Optical density at 405 nm in sera from 33 control subjects, 16 patients with Hashimoto's disease, and 33 patients with Graves' disease. *Bars*, median values. Mean ± SE values were .098 ± .009 for control subjects, .110 ± .016 for Hashimoto's disease patients, and .172 ± .024 for Graves' disease patients ($P$ = .004 compared with normal).**

patients positive for insulin autoantibody by ELISA had evidence of another autoimmune disorder.

Sera from 16 patients with Hashimoto's disease were similarly tested and resulted in a mean (±SE) optical density of .110 ± .016 (range .010–.267; Fig. 1). Only one of the 16 patients had an optical density >.2. These binding values were not significantly different from normal ($P$ = .45) or from Graves' disease patients' values ($P$ = .23).

**Insulin-receptor–binding studies.** Thirty-three patients with Graves' disease and 15 patients with Hashimoto's disease were screened for the presence of insulin-receptor antibodies by binding-inhibition assay. Known positive sera had ~5% of normal binding. All of the test sera exhibited at least 90% of control binding, indicating that none of the patients with autoimmune thyroid disease had evidence of insulin-receptor–antibody activity.

**Cytoplasmic islet cell antibodies.** Serum from seven patients with Graves' disease and an optical density >.200 in the insulin-binding ELISA were screened for the presence of cytoplasmic islet cell antibodies. None of these patients was positive for islet cell antibodies. Additionally, one of the serum samples of a patient with Hashimoto's disease and an optical density of .193 was negative for cytoplasmic islet cell antibodies.

## DISCUSSION

In this study, insulin-binding antibodies were present in 10 of 33 patients with Graves' disease and in 1 of 16 patients with Hashimoto's disease; no patient with autoimmune thyroid disease had insulin-receptor antibodies. None of 8 insulin-antibody–positive patients had islet cell antibodies. These data confirm that the autoantibody response in patients with autoimmune thyroid diseases extends beyond thyroid antigens (1,3) and support the concept that these disorders may represent a generalized polyclonal B-lymphocyte response (11,12). The fact that insulin-binding antibodies were noted is particularly interesting given the frequent coincidence of diabetes mellitus and autoimmune thyroid diseases (13).

Moreover, there have also been reports of spontaneous hypoglycemia in patients with hypothyroidism or Graves' disease (14,15). Hirata et al. (14) described a patient with Graves' disease who had severe episodes of hypoglycemia, elevated serum insulin levels (up to 35,280 μU/ml), and the presence of insulin-binding antibodies. These metabolic abnormalities were discovered 3 wk after the diagnosis of Graves' disease and were still present 1 yr later, persisting after Graves' disease had been treated. It is unknown whether the antibodies that bind to insulin represent the identical subpopulation of antibodies recognizing thyroid antigens that are found in patients with autoimmune thyroid disease. It is conceivable that insulin and thyroid antigens have cross-reacting epitopes, to which subsets of B-lymphocytes generate antibodies (16). Furthermore, it is unknown whether these insulin-binding antibodies are capable of causing metabolic alterations by either inhibiting or exaggerating insulin action, although, as noted above, such a case has been reported. Some of our patients may have false-negative assays for insulin-binding antibodies, because these antibodies could have escaped detection if they were bound in an immune complex. As noted, however, none of our patients had anti-insulin-receptor antibodies.

Insulin autoantibodies are known to occur spontaneously in patients without a prior history of insulin injections, and furthermore, insulin-binding antibodies frequently occur before the onset of type I diabetes (7,17–19). Wilkin et al. (8) reported that 38% of newly diagnosed type I diabetic subjects and 47% of their unaffected identical twins had insulin-binding autoantibodies. One difference between these earlier data and ours is that patients who develop type I diabetes also frequently have antibodies against the islet cell, whereas our patients with autoimmune thyroid disease do not (9,18–20). Additionally, insulin-receptor antibodies were not found in patients with insulin autoantibodies, and we could not confirm an earlier report that insulin-receptor antibodies may occur as an anti-idiotype in patients positive for insulin antibodies (20).

The insulin antibodies detected in this study are present in patients with both active and treated Graves' disease, yet they are not associated with clinically significant abnormalities in glucose tolerance. Long-term studies are required to examine the nature of the insulin-binding antibodies occurring in the setting of autoimmune thyroid disease as well as their relationship to the potential development of glucose intolerance.

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