Thyroid cytotoxic antibodies in atrophic and goitrous autoimmune thyroiditis

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It is unknown whether in chronic lymphocytic thyroiditis the goitrous (Hashimoto’s thyroiditis) and atrophic forms (primary myxedema) are variants of the same disease or different pathogenic entities. Conventional thyroid-related autoimmune parameters are unable to separate both diseases serologically. It is assumed that cellular and humoral cytotoxic events induce gland atrophy and thus should be detectable more often in non-goitrous than goitrous autoimmune thyroiditis. We determined antibody-dependent cell-mediated cytotoxicity in 67 patients with autoimmune thyroiditis, using a 51chromium-release assay against human thyroid cells. Thyroid volume had been measured by ultrasonography. Other thyroid-specific antibodies, like TSH binding-inhibiting antibodies, TSH function-blocking antibodies, thyroglobulin antibodies and thyroid peroxidase antibodies, were determined. Cytotoxic antibody activity was 20.5% (median, range 0–54.5%) in patients with autoimmune thyroiditis and 8.3% (median, range 0–18.4%) in controls (p < 0.0001). Analysis of cytotoxicity regarding thyroid size showed a high incidence of cytotoxic antibodies in atrophic disease (median thyroid volume 6 ml), where cytotoxic antibodies were detectable in 80% versus 39% (x² = 9.6; p < 0.0001) in goitrous disease (median thyroid volume 36 ml). The specific lysis of 30% (median: 95% confidence limit 23.9–32.9) in non-goitrous thyroiditis patients was significantly higher than in goitrous patients (16.9%; 95% confidence limit 13.2–20.4) (p = 0.0006). Prevalence of thyroglobulin and thyroid peroxidase antibodies were equally distributed in both groups, with slightly higher levels of thyroid peroxidase antibodies in goitrous thyroiditis (p < 0.05). Both TSH binding-inhibiting and TSH function-blocking antibodies were rarely positive in either atrophic or goitrous disease. Our study shows for the first time a striking association of thyroid cytotoxic antibodies with the atrophic variant of autoimmune thyroiditis. We suggest that the occurrence of cytotoxic antibodies in the pathogenesis of chronic lymphocytic thyroiditis is the decisive event that favors the development of the atrophic rather than goitrous form of the disease.

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There is now considerable evidence that chronic lymphocytic thyroiditis is an autoimmune thyroid disease. Although it appears that the different forms of autoimmune thyroiditis may be caused by closely related immunological disturbances, some features of the diseases still remain unexplained. It is unknown why some patients with autoimmune thyroiditis develop a goiter with intensive lymphocytic infiltration and others an atrophic gland with minimal lymphocytic infiltration but extensive fibrosis. It has been emphasized that genetic differences may exist between goitrous Hashimoto’s thyroiditis and atrophic thyroiditis (primary myxedema). An increased association of HLA-B8 and HLA-DRw3 with atrophic thyroiditis and HLA-DR5 with goitrous disease was found (1–3). Furthermore, TSH receptor-blocking antibodies are found more often in atrophic than in goitrous thyroiditis (4–8). However, the prevalence of various types of TSH receptor-blocking antibodies in patients with atrophic thyroiditis is too low to be the sole factor for atrophy in the majority of these cases.

The histological features of atrophic autoimmune thyroiditis indicate that cell destruction and replacement of thyroid follicles by connective tissue are the main events. This report provides evidence that cytotoxic antibodies are an important immunological event favoring the development of atrophic rather than goitrous disease.

Subjects and methods

Subjects

Sixty-seven patients, 63 women and four men, aged 14–79 years (mean 53 years) with autoimmune thyroiditis were studied. Forty-one patients, mostly with non-goitrous thyroiditis, were recruited from Berlin, Germany, and 26 with a hypertrophic thyroiditis from

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Herlev, Denmark. The diagnosis was based on findings of elevated TSH values, significantly increased titers of thyroid peroxidase (> 1.3 \times 10^8 \text{mU/L}) and thyroglobulin antibodies (> 1 : 80), typical hypochoic ultrasound images and, in some cases, biopsy evidence of lymphocytic infiltration. The iodine intake in Denmark and Germany is borderline deficient with a urinary iodine excretion of 50–100 \mu g/day (9, 10). To avoid repeatedly freezing and thawing, sera from both centers were collected and frozen in aliquots. Frozen sera were shipped to Berlin on dry ice.

Thyroid volume was determined in all patients by real-time sonography according to the mathematical procedure described previously (9). The upper limit of normal thyroid volume is 20 ml for women and 25 ml for men. A thyroid volume of < 15 ml was regarded as non-goitrous or atrophic and > 25 ml as hypertrophic. Sixty-four patients were hypothyroid or subclinically hypothyroid, and four were euthyroid. At the time of investigation 49 patients were euthyroid on L-thyroxine medication for a various time period (2 months–30 years), 17 hypothyroid and one patient euthyroid without treatment. Controls were 61 normal subjects, 53 women and eight men, aged 24–83 years (mean, 50 years) who did not have a history of thyroid disease or show significant titers of thyroid antibodies.

**Thyroid cells**

Surgically obtained thyroid-tissue specimens from patients with multinodular goiter (blood group O) were finely minced and enzymatically isolated by incubation in 0.5% collagenase (Boehringer, Mannheim, Germany). After a 45-min incubation at 30°C, the supernatant was decanted through a screen and mixed with Iscove's modified Dulbecco's medium (Seromed, Berlin, Germany) containing 10% fetal calf serum (FCS) (Seromed), 10^5 \text{mU/L} TSH (Organon, Munich, Germany). 10 \text{mU/L} penicillin and 100 mg/l streptomycin (Seromed). After three washes, cells were diluted to 5 \times 10^6 cells/ml in Iscove's medium containing 10% FCS, antibiotics and 7.5% dimethyl sulfoxide (Sigma, München, Germany). The cell solution was transferred to cryovials, frozen and stored in liquid nitrogen until used for the experiments.

**Determination of antibody-dependent cell-mediated cytotoxicity (ADCC)**

Antibody-dependent cell-mediated cytotoxicity was determined as described previously (11). The frozen cells were replated by rapidly warming the vials at 37°C and transferring them in prewarmed Iscove's medium into 75-cm² flasks. After 3 days of culture, the cells had reached confluency and were transferred in suspension by incubation with 10 ml of trypsin/EDTA solution (0.05%/0.02%) (Seromed) for 5 min at 37°C. After several washes, 2 \times 10^6 thyroid cells were incubated for 1 h with 100 µCi of Na_2^{51}CrO_4 (Behring AG, Marburg, Germany), washed twice and diluted to 10^5 cells/ml. Fifty microtiter of radiolabelled cells (5 \times 10^3 cells/well) then were incubated in triplicate with 100 µl of 1 : 10 diluted heat-inactivated serum in V-bottom microtiter plates (Flow Laboratories, McLean, VA, USA) at 37°C and 5% CO₂ in a water-saturated incubator. In previous studies optimal results were yielded with a serum dilution of 1 : 10. After 1 h of incubation, the supernatant was removed and the cells were washed once with medium. Then 100 µl of effector mononuclear cells (1.25 \times 10^5) from a normal subject were added, yielding an effector/target cell ratio of 25 : 1. After 18 h of incubation, an aliquot of the supernatant was aspirated and the radioactivity of the test sample was measured in a gamma counter (cpmexp). Samples for measuring the 100% value (cpmmax) and the unspecific lysis (cpmunspec) contained medium instead of lymphocytes. The unspecific release was determined from an aliquot of the supernatant and the maximal release from an aliquot of the incubation mixture. Specific lysis was calculated according to the following formula:

\[
\text{% Specific lysis} = \frac{\text{cpm}_{\text{exp}} - \text{cpm}_{\text{unspec}}}{\text{cpm}_{\text{max}} - \text{cpm}_{\text{unspec}}} \times 100
\]

Cytotoxicity was considered positive if the specific lysis of the patient's serum was above the 95th percentile of the control sera determined in the same assay. To avoid influences on the ADCC results due to various components in the test system, all sera were analyzed using the same target and effector cells. Nevertheless, the data were reproducible using different target and effector cell sources except with thyrocytes from Graves' disease patients exhibiting high unspecific background. Cytotoxicity values given in this paper are the mean of two measurements. The intra-assay variance was 10% and the interassay variance was 20%.

For control experiments, human liver tumor cells (Chang cells) were cultured, harvested and radiolabeled as described for thyroid cells. The radiolabeled cells were used as targets in the cytotoxicity assay, which was performed exactly as described for the ADCC assay with human thyroid cells.

**Thyrotropin function-blocking antibodies (TB-ab)**

Thyroid cells were thawed and cultured to confluency as described previously, and 2 \times 10^6 cells/well were plated in 96-well flat-bottom microtiter plates (Nunc, Wiesbaden, Germany) and cultured for 1–3 days. Then normal or patient IgG (1 g/l), 200 mU/l hTSH (Organon, Munich, Germany) and 0.5 mmol/l methylxanthine were added. After a 24-h incubation, the supernatant was harvested and the cAMP concentration determined using a commercial kit (DuPont, Dreieich, Germany). A TSH stimulation curve had been established in previous
Based on ultrasonographically determined thyroid volume, patients were grouped as having non-goitreous (N = 34) or hypertrophic (N = 33) autoimmune thyroiditis. Thyroid size in non-goitreous patients was 6.0 ml (median, range 2.8–13.0 ml) versus 36 ml (median, range 25.0–282.0 ml) in goitreous patients (p < 0.0001) (Fig. 1). Both groups were comparable with regard to number, sex, age and thyroid function at the time of diagnosis (Table 1).

Antibody-dependent cell-mediated cytotoxicity, expressed as specific lysis, was 20.5% (median, range 0–54.5%) in serum of patients with autoimmune thyroiditis versus 8.3% (median, range 0–18.4%) in that of 61 controls (p < 0.0001). Altogether, cytotoxic antibodies were detectable (values > 17.7% = 95th percentile of % specific lysis of normal sera) in 41 of 67

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<th>Table 1. Clinical and immunological parameters in patients with atrophic and hypertrophic autoimmune thyroiditis.</th>
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* TPO-ab: thyroid peroxidase antibodies; TG-ab: thyroglobulin antibodies; TBI: TSH binding-inhibiting antibodies; TB-ab: TSH function-blocking antibodies; ADCC: antibody-dependent cell-mediated cytotoxicity; †p < 0.05 and ‡p < 0.0001.
patients (61%). Analysis of the data with regard to thyroid volume showed that cytotoxic antibodies were detectable more frequently in non-goitrous (27 of 34 patients; 80%) than in goitrous autoimmune thyroiditis (13 of 33 patients: 39%) (\(x^2 = 9.6; p < 0.0001\)) (Table 1) and exhibited significantly higher antibody activity in the former group. Sera of patients with atrophic disease induced a specific lysis of 30% (median, range 7.2–54.5%) (95% confidence limit 23.9–32.9) versus 16.9% (median, range 0–40.8%) (95% confidence limit 13.2–20.4) for those with gland hypertrophy (\(p = 0.0006\)) (Fig. 2). The actual thyroid status at the time of blood collection had no influence on the cytotoxicity data.

None of the sera exhibiting a positive cytotoxic effect against thyroid cells induced cell damage of Chang liver cells, excluding an unspecific effect due to toxic substances in the sera. Cytotoxicity with Chang cells was 20.9 ± 6.6% in patients with hypertrophic disease and 19.9 ± 7% in patients with atrophic gland, and did not differ compared to normal controls (18.6 ± 8.1%) (\(p = NS\)).

As described previously, no association was found between cytotoxic antibody activity and titers of TG-ab and TPO-ab (13). Separate analysis of % specific lysis and titers of TPO-ab in atrophic and hypertrophic patients also gave no correlation between these parameters (r = –0.14 and r = –0.29 in hypertrophic and atrophic patients, respectively). There was only a tendency towards an inverse correlation between the cytotoxic antibody activity and the thyroid volume (\(p = 0.6\)) (data not shown).

Apart from cytotoxicity, no other thyroid-related autoimmune parameter showed similarly significant differences between the two study groups. Thyroglobulin antibodies were equally detectable at 53% and 52% in atrophic and hypertrophic disease, respectively, with no difference in their titer levels.

Thyroid peroxidase antibodies were more frequently positive at 82% and 91%, respectively, titer levels being slightly higher in goitrous (median 7 \(\times 10^9\) mU/l) than in non-goitrous thyroiditis (median 2.1 \(\times 10^9\) mU/l) (\(p < 0.05\)) (Fig. 3). Thyrotropin binding-inhibiting antibodies were detectable in only three patients with an atrophic gland and negative in all those with a hypertrophic gland. Similarly, antibodies that block the TSH-induced cAMP increase (TB-ab) were positive in only a small number of patients: four with non-goitrous and one with goitrous disease (Table 1).

**Discussion**

Although the pathogenetic mechanism is an autoimmune process in both goitrous (Hashimoto’s thyroiditis) and atrophic thyroiditis (primary myxedema), it remains unclear why the gland enlarged in some immune thyroiditis patients and remained normal or atrophic in others. Histologically, atrophic autoimmune thyroiditis involves progressive shrinking of the thyroid gland, loss of epithelium, lymphocytic infiltration and replacement of the glandular structure by fibrous tissue. It has been suggested, therefore, that thyroid cell destruction is initiated by antibody- and cell-mediated mechanisms. We described recently a \(^{51}\)Cr release assay with human thyroid cells for measurement of ADCC in autoimmune thyroiditis (11). Our present data demonstrate for the first time a significant association of cytotoxic antibodies with atrophic thyroiditis. The occurrence of cytotoxic antibodies in the pathogenetic cascade of autoimmune reactions against thyroid tissue might be the crucial step that triggers the development of atrophic disease. Lack of ADCC and/or other yet unknown mechanisms might be responsible for the intense lymphocytic infiltration, goiter formation and hypothyroidism in goitrous thyroiditis.

Although we and others found significantly increased cytotoxic activity in Hashimoto’s sera, these results
were not confirmed unanimously (11, 13–16). Applying an ADCC method with thyroid target cells, Sack et al. recently found normal cytotoxicity with lymphocytes from patients as well as with sera from patients with Hashimoto’s disease and lymphocytes from normals as effector cells (16). In light of our present data, this apparent discrepancy could be explained by patient selection in their study investigating goitrous rather than atrophic Hashimoto’s disease.

Earlier data of our group and others suggested that cytotoxic antibody activity is located in the microsomal antibody fraction (11, 14, 15). Extending our investigations we could not confirm these data, which can be explained by biases due to small sample size. We determined ADCC and TPO-ab in more than 60 patients with autoimmune thyroid disease and did not find a correlation between these parameters (13). Furthermore, the addition of purified TPO to cytotoxic antibody-positive serum with or without TPO-ab positivity did not influence the results in the cytotoxicity assay, thus suggesting two separate antigen–antibody entities (13).

No significant correlation could be found between cytotoxicity levels and thyroid volume. This is not surprising in view of the temporal influence on antibody activity in autoimmune thyroiditis. Almost all patients were hypothyroid at the initial clinical and chemical investigation. However, when being investigated for our study, 49 patients (73%) had been euthyroid on l-thyroxine medication for different time periods. It is well known that microsomal antibody titers decrease during long-term thyroxine treatment, which seems to be valid for cytotoxic antibody activity as well (17).

In Hashimoto’s thyroiditis, infiltrating lymphocytes consist predominantly of cytotoxic T cells, which raises the question of the role of T-cell-mediated cytotoxicity in thyroid cell destruction (18–22). CD8+ T-cell clones, isolated from infiltrating lymphocytes, were found to express natural killer cell activity (20, 21). MacKenzie and Davies (23) isolated a cytotoxic T-cell clone that induced autologous thyroid cell destruction but showed no cytotoxic activity to allogeneic thyrocytes. The systematic analysis of T-cell cytotoxicity in an autologous system is not possible owing to methodological reasons, so there will still be a lack of sufficient knowledge about this immune reaction in atrophic and hypertrophic autoimmune thyroiditis, even in the future. Recent data on insulin-dependent diabetes showed an inverse relation between humoral and cellular immunity to glutamic acid decarboxylase (GAD) (24). The authors speculated that a strong T-cell activation by the antigen is correlated with rapid development of the disease and that high concentrations of GAD antibodies are associated with slower progression. Whether this dichotomy is responsible also for the differentiation in atrophic and hypertrophic autoimmune thyroid disease has to be elucidated.

It has been suggested that TSH receptor antibodies, which inhibit the receptor binding or function of TSH, may contribute to the diversification of immune thyroiditis. In almost all studies, receptor antibodies were detectable rarely in patients with autoimmune thyroiditis and, if at all, in those with the atrophic rather than the goitrous form (4–8, 25–27). Our study demonstrated a very low percentage of TSH binding-inhibiting and TSH function-blocking antibodies, which is in agreement with the aforementioned studies and confirms that receptor antibodies do not contribute to the clinical manifestation of the disease in two different forms. Excluding patients with positive values for TB-ab and TBI from the analysis, the statistical significance of cytotoxic antibody values in the atrophic vs hypertrophic group remained unchanged (p = 0.0006).

It has not been clarified as yet whether or not goitrous and atrophic autoimmune thyroiditis are indeed the same disease, with primary myxedema as the endstage of Hashimoto’s thyroiditis. Genetic factors have been assumed as determinants but several studies yielded variable results: HLA-B8 and -DR3 were found to be increased with atrophic and HLA-DR3, -DR4 and -DR5 were increased with hypertrophic thyroiditis (1–3, 28–30). Recent investigations by Badenhoop et al. using molecular analysis of the HLA-D region genes revealed a closer association with the HLA-DQ7 locus (28). Applying the same molecular approach we found similar results in non-goitrous thyroiditis, with an increased association with HLA-DR5 and -DQ7, and concluded that the susceptibility to one of the diseases is not determined by genetic factors (31). Other autoimmune parameters, like antithyroglobulin and anti-TSH receptor antibodies, exhibited higher frequencies and higher titers in atrophic disease but there was still a high degree of overlap (32, 33).

Knowledge is limited concerning the spontaneous course of euthyroid Hashimoto’s thyroiditis, because almost all patients are treated with thyroxine (32, 34). Our clinical observation that almost none of our patients with non-goitrous autoimmune thyroiditis observed or remembered having a goiter before the onset of hypothyroidism suggests that cytotoxic antibodies are an early event rather than a consequence of pre-existing goitrous Hashimoto’s thyroiditis. Our results of unequal distribution of cytotoxic antibodies in goitrous and atrophic autoimmune thyroiditis favor the hypothesis that the occurrence of these antibodies in the pathogenetic cascade of autoimmune reactions determines the development of atrophic rather than hypertrophic disease.

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