Thyroid peroxidase/microsomal antibodies are not identical with thyroid cytotoxic antibodies in autoimmune thyroiditis

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Abstract. Cytotoxic activity in sera of patients with Hashimoto's thyroiditis was measured with an antibody-dependent cell-mediated cytotoxicity assay. Cytotoxicity was determined in a 51chromium release assay using human thyroid cell targets incubated with heat-inactivated serum or IgG from patients with Hashimoto's thyroiditis. Effector cells were obtained from peripheral mononuclear cells of normal subjects. Cytotoxicity was significantly increased in patients with Hashimoto's thyroiditis (median specific lysis 20.2%, range 2.1-58.8) compared with normals (median specific lysis 8.1%, range 0-19.5; p<0.0001). The amount of percent specific lysis did not correlate with the titres of microsomal antibodies determined by different methods: passive hemagglutination technique (r=0.2), enzyme immunoassay with microsomal antigen (r=0.16), and radioimmunoassay for thyroid peroxidase antibody (r=0.02). The cytotoxic activity was located in the IgG fraction, both in microsomal antibody positive and negative sera. After pre-incubation of microsomal antibody/thyroid peroxidase antibody positive or negative sera with purified thyroid peroxidase followed by analysis in the antibody-dependent cell-mediated cytotoxicity assay, cytotoxicity decreased in only 2 cases but was unchanged in the remaining sera. Western blot experiments with solubilized thyroid membranes and immunoblotting with cytotoxic-positive/microsomal antibody negative sera showed no binding to thyroid peroxidase. Our data suggest that cytotoxicity in sera from patients with Hashimoto's thyroiditis is not mediated by antibodies against thyroid peroxidase, but by antibodies not yet identified.

Since the detection of microsomal antibodies in Hashimoto's thyroiditis and the association of cytotoxic antibody activity with the microsomal antibody fraction, it has been generally accepted that the presence of these antibodies is an indicator of thyroid cell destruction (1-4). Nevertheless, clinical follow-up studies showed that in some patients microsomal antibodies are present for many years without development of hypothyroidism (5,6). Preliminary data of our laboratory suggest that there is no correlation between cytotoxic antibody activity and titres of microsomal antibodies (7). In this paper we add further evidence for the diversity of cytotoxic and microsomal/thyroid peroxidase antibodies.

Patients and Methods

Sixty-seven patients with autoimmune thyroiditis were studied, 57 women and 10 men, aged 21-83 years (mean 55). The diagnosis was established by findings of elevated TSH, significant titres of thyroid microsomal antibodies (≥1:400), thyroglobulin antibodies (≥1:100), typical hyporeflexive image by ultrasonography, and, in some cases, by biopsy evidence of lymphocytic infiltration. Sixty-one normal subjects were included in the study, 53 women and 8 men, aged 27-64 years (mean 45), without significant titres of thyroid antibodies and no history of thyroid disease.

Thyroid cells

Thyroid tissue obtained from surgical specimens of patients with multinodular goitre (blood group 0) was finely minced and enzymatically isolated by incubation in 0.5% collagenase (Boehringer, Mannheim, FRG). After 45 min of incubation at 37°C, the supernatant was decanted through a screen and mixed with Iscove's modified Dulbecco's medium (Seromed, Berlin, FRG) contain-
ing 10% fetal calf serum (FCS) (Seromed), 10 U/l bTSH (Organon, Munich, FRG), 100 kU/l penicillin and 100 mg/l streptomycin (Seromed). After three washes, the cells were diluted to 5 x 10^6/ml in Iscove’s medium containing 10% FCS, antibiotics, and 7.5% dimethylsulfoxide (Sigma, München, FRG). The cell solution was transferred to cryovials, frozen and stored in liquid N2 until used for the experiments.

**Determination of antibody-dependent cell-mediated cytotoxicity**

Antibody-dependent cell-mediated cytotoxicity (ADCC) was determined as previously described (8). The frozen cells were replated by rapidly warming the vials at 37°C and transferring them to preheated Iscove’s medium in 75 cm² flasks. After three days of culturing, the cells had reached confluency and were transferred in suspension by incubation with 10 ml trypsin/EDTA solution (0.05%/0.02%) (Seromed) for 5 min at 37°C. After several washes, 2 x 10^6 thyroid cells were incubated for 1 h with 100 μCi Na^25^CrO_4 (Behring AG, Marburg, FRG), washed twice and diluted to 10^6 cells/l. Fifty μl radiolabelled cells (5 x 10^5) well corresponding to 1500-2500 cpm; specific radioactivity 37-45 kBq 31Cr/10^6 thyrocytes) were then incubated in triplicate with 100 μl 1:10 diluted heat-inactivated serum or 100 μl IgG (1 g/l) in microtiter plates (Flow Laboratories, McLean, VA) at 37°C and 5% CO_2 in a water-saturated incubator. After 1 h of incubation the supernatant was removed and the cells were washed once with medium. Then 100 μl effector mononuclear cells (1.25 x 10^6) from a normal subject were added, resulting in an effector:target cell ratio of 25:1. After 18 h of incubation, an aliquot of the supernatant was aspirated and the radioactivity was measured in a gamma counter (cpm_{exp}). Samples for the determination of the 100% value (cpm_{max}) and nonspecific lysis (cpm_{nonspec}) contained medium instead of lymphocytes. The nonspecific release was determined by counting an aliquot of the supernatant, and maximal release was determined in an aliquot of the incubation mixture. Specific lysis was then calculated according to the following formula:

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\text{% specific lysis} = \frac{\text{cpm}_{\text{exp}} - \text{cpm}_{\text{nonspec}}}{\text{cpm}_{\text{max}} - \text{cpm}_{\text{nonspec}}} \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.

**Addition of thyroid peroxidase**

Fifty μl serum from patients with Hashimoto’s thyroiditis (N=9) in whom the percent specific lysis was previously found to be elevated was pre-incubated with purified thyroid peroxidase (TPO) (final concentration 66 and 100 mg/l) at 37°C for 90 min. The sera were then centrifuged at 6000 x g and the supernatant added in the ADCC assay. The TPO-ab titres of the sera, determined by TPO-ab radioimmunoassay, varied between 0 and >10^7 U/l.

**IgG preparation**

The IgG fraction was isolated from serum samples by means of affinity chromatography on protein A-Sepharose CL-4B columns (Pharmacia, Uppsala, Sweden) according to the instructions of the manufacturer. Peak fractions detected by spectrophotometry (280 nm) were combined and protein concentration adjusted to 1 g/l.

**Effector cells**

Fresh mononuclear cells were obtained from heparinized blood of a normal adult by means of Ficoll (Seromed) density centrifugation. The mononuclear cells were washed and diluted with medium to 1.25 x 10^6/l.

**Microsomal/thyroid peroxidase and thyroglobulin antibodies**

Serum microsomal antibodies (Mi-ab) were determined by passive hemagglutination technique (Thymune M, Wellcome Diagnostics, Kent, UK) and by an enzyme immunoassay in which purified microsomal antigen was bound to a solid phase (Thyr Mic-IgG ELA, Pharmacia). Thyroid peroxidase antibodies were measured by a radioimmunoassay against solid-phase-bound monoclonal anti-TPO-antibodies (DYNOnest, Henning Co, Berlin, FRG), Mi-ab titres of ≥1:400, >16 kU/l, >100 kU/l, determined by passive hemagglutination, ELISA, and TPO-ab radioimmunoassay, respectively, were defined as positive. Thyroglobulin antibodies (Tg-ab) were measured by passive hemagglutination technique (Thymune T, Wellcome Diagnostics). Tg-ab titres ≥1:100 were considered positive.

**Thyroid membrane preparation**

Thyroid tissue obtained at operation from patients with Graves’ disease or multinodular goitre was minced and homogenized in an Omnimixer (Sorvall Instruments, DuPont, Wilmington, USA). The homogenate was centrifuged at 1000 x g for 5 min. The supernatant was collected and spun at 100 000 x g for 1 h at 4°C. The pellets were washed four times in 10 mmol/l TRIS/HCl, 150 mmol/l NaCl buffer (TBS) and resuspended in 30 ml of the same buffer. The suspension was layered on a sucrose gradient and centrifuged at 100 000 x g for 2.5 h. The layer at the interface between 24 and 40% sucrose was collected and washed with TBS. The membranes were solubilized at 65°C for 15 min in 0.009 mmol/l TRIS/HCl buffer containing 2.5% SDS and 2.25 mmol/l urea. The protein concentration was approximately 10 g/l.

**Immunoblotting**

SDS-PAGE was performed using a 5% polyacrylamide gel under non-reducing conditions. Routinely, 50-100 μg membrane protein were electrophoresed. Proteins re-
solved by electrophoresis were electrophoretically transferred onto a nitrocellulose sheet in 25 mmol/l TRIS/HCl buffer with 150 mmol/l glycine and 20% methanol. After transfer, nitrocellulose sheets were incubated in TBS with 0.5% Tween (TTBS) and 3% gelatine to block unspecific binding. The sheets were incubated with serum samples diluted to 1:250 with TTBS and 1% gelatine at room temperature. After 2 h the sheets were washed four times with TTBS with 1% gelatine. Bound antibodies were visualized by incubation for 1 h with phosphatase-bound goat anti-human IgG (Sigma, Munich, FRG). After washing four times with TTBS and once with substrate buffer, substrate was added and incubation was continued until bands were visible (2-30 min). For preparation of the substrate, 5 mg 5-bromo-4-chloroindoxylphosphate (Sigma) (BCIP) and 1 mg nitroblue tetrazolium (Sigma) (NBT) were each dissolved in 1 ml H2O. A mixture of 9 ml carbonate buffer (100 mmol/l, pH 9.5), 1 ml NBT and 0.1 ml BCIP was used for the reaction.

Statistical analysis
Data were assessed for significance using the unpaired Mann-Whitney U-test. A p-value <0.05 was considered significant.

Results

Antibody-dependent cell-mediated cytotoxicity in Hashimoto's thyroiditis
ADCC was positive in 42 (63%) of 67 investigated patients with Hashimoto's thyroiditis. The median specific lysis was 20.2% (range 2.1-58.8) compared with 8.1% (range 0-19.6) in the normal controls (p=0.00001) (Fig. 1). Values above the 95th percentile (≥15.4%) were regarded as positive. The values of the percent specific lysis did not correlate with the duration of the disease.

Correlation of cytotoxicity with titres of microsomal/TPO antibodies
Significantly increased titres of microsomal antibodies were found in 72% of the patients with Hashimoto's thyroiditis (range 1:400-1:25600). Sera were available from 47 patients in whom, in addition to the passive hemagglutination technique, the Mi-ab titres were determined by enzyme immunoassay and the TPO-ab titres by radioimmunoassay. The enzyme immunoassay for Mi-ab determination yielded positive results in 37 of the 47 patients (79%), and 38 of the 47 patients (81%) were positive in the radioimmunoassay for the measurement of TPO-ab. No correlation could be found between the titres of Mi-ab and TPO-ab determined by passive hemagglutination, enzyme immunoassay or TPO-ab RIA and the activity of the cytotoxic antibodies (r=0.2, r=0.16 and r=0.02, respectively) (Fig. 2a-c). There was likewise no correlation between the cytotoxicity and the titres of thyroglobulin antibodies (r=0.00).

Characterization of the cytotoxic activity
To exclude an influence of unspecific serum components on thyroid cell lysis, IgGs were isolated by protein-A sepharose chromatography and added to the cytotoxicity assay instead of serum. IgGs from 16 sera with positive and 4 sera with negative Mi-ab and Tg-ab titres were simultaneously tested in the ADCC assay. The median specific lysis in the Mi-ab/Tg-ab positive group was 25% with addition of serum and 21.6% with addition of IgG, in the
Fig. 2.
Correlation between percent specific lysis and titres of microsomal antibodies determined simultaneously by 3 different methods. a) passive hemaggulination (r=0.2); b) microsomal antibody ELISA (r=0.16); c) TPO-ab RIA (r=0.02).

Fig. 3.
Cytotoxicity determined by addition of serum and IgG (1 g/l) in the antibody-dependent cell-mediated cytotoxicity (ADCC) assay. The median specific lysis in the microsomal antibody (Mi-ab) positive group was 25% (range 17.5-43.6, N=16) with serum and 21.6% (range 10.6-45.3) with IgG, and in the Mi-ab negative patients 36.9% (range 29.9-46.1, N=4) and 33.6% (range 22.5-48.2), respectively. In both groups (Mi-ab positive and negative) the cytotoxic activity was found to be located in the IgG fraction, which proves that cytotoxic activity in Mi-ab negative sera is also mediated by an IgG.

Mi-ab/Tg-ab negative group 36.9 and 33.6%, respectively (Fig. 3).

To investigate in more detail whether the cytotoxic antibody is different from the TPO antibodies, sera with positive and negative titres of TPO-ab, but positive results for cytotoxicity, were pre-incubated with purified TPO for 2 h and then added to the ADCC assay. The cytotoxic activity remained unchanged in 7 of 9 sera, irrespective of whether they had a positive or a negative result for TPO-ab (Fig. 4). Only 2 cases showed a significant decrease in cytotoxicity after pre-incubation with TPO, both with low (130 kU/l) or undetectable titres for TPO-ab.

Immunoblotting following SDS-PAGE
Western blot experiments following SDS polyacrylamid gel electrophoresis were performed to show binding of the cytotoxic antibody to human thyroid membranes. Sera of patients with high cytotoxic activity in the ADCC assay and positive as well as negative titres for Mi-ab and Tg-ab were tested
Microsomal antibodies were measurable at a titre of 1:25600 (passive hemagglutination) in serum E, which served as positive control for the binding to the microsomal antigen.

Discussion

Two different immunological reactions were used in the past to determine antibody-mediated cytotoxicity in patients with Hashimoto’s thyroiditis: 1. antibody-dependent complement-mediated cytotoxicity and 2. antibody-dependent cell-mediated cytotoxicity.

Using an antibody-dependent complement-mediated cytotoxicity assay, Pulvertaft et al. (1,2) described for the first time in sera from patients with Hashimoto’s thyroiditis a cytotoxic factor which induced lysis of cultured thyroid cells. Further investigations showed that this factor is located in the microsomal antibody fraction, that the effect is abolished by heat-inactivation to 56°C, and that it can be reinstalled by the addition of fresh complement (3,4). Absorption experiments with thyroglobulin proved that cytotoxic and thyroglobulin antibodies are not identical (1,9). Some authors found a positive cytotoxic effect in sera of patients with Hashimoto’s thyroiditis, whereas their titres of microsomal antibodies were negative and vice versa (1,9). Chandler et al. (10) found no correlation between complement-mediated cytotoxicity and titres of microsomal antibodies and suggested that the cytotoxic effect is mediated by a factor independent of the microsomal antibodies. In contrast, Khoury et al. (11) demonstrated thyroid cell lysis with microsomal antibody positive sera, and recently Wadeleux et al. (12) likewise showed thyroid cell damage owing to antibody-dependent complement-mediated cytotoxicity which correlated to the titres of the microsomal antibodies. Although the data of the studies are not uniform, it has been generally accepted that the cytotoxic factor is located in the microsomal antibody fraction.

In accordance with the results of complement-mediated cell lysis, studies analysing cytotoxic effects by means of antibody-dependent cell-mediated cytotoxicity assays also showed a higher frequency of positive results in patients with Hashimoto’s thyroiditis; unfortunately, the titres of the microsomal antibodies were not simultaneously reported (13-15). Earlier data obtained in our laboratory by applying an ADCC assay with cultured

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**Fig. 4.**
Influence of pre-incubation with increasing concentrations of thyroid peroxidase (TPO) for 2 h on percent specific lysis in sera of patients with Hashimoto’s thyroiditis. Values at the curves indicate the TPO-ab levels.

**Fig. 5.**
SDS-PAGE of solubilized thyroid membrane fractions and immunoblotting of sera from patients with Hashimoto’s thyroiditis. Lane A: normal serum; lane B: Cytotoxicity and microsomal antibody (Mi-ab)/thyroglobulin antibody (Tg-ab) positive serum (specific lysis 43.6%; Mi-ab 1:400; Tg-ab 1:2560); lane C and D: Cytotoxic-positive, Mi-ab/Tg-ab negative sera (specific lysis 33.9 and 32%, respectively, Mi-ab/Tg-ab negative); lane E: Mi-ab positive control serum (Mi-ab titre 1:25600). Mag = microsomal antigen. Tg = thyroglobulin.
human thyroid cells as targets indicated a simultaneous occurrence of cell lysis and microsomal antibodies (8). In a larger group of patients, however, we could no longer detect any correlation between cytotoxicity and microsomal antibody activity, which emphasizes the biases associated with investigating small collectives. These results are in agreement with previously published data of patients with Graves' disease in whom we were likewise unable to find a connection between cytotoxicity and microsomal antibody activity (7).

The microsomal antigen has now been identified as thyroid peroxidase (16,17). To exclude the influence of false-negative Mi-ab determinations by the passive hemagglutination technique, we measured the antibody concentrations in the same sera by a highly sensitive ELISA technique and TPO-ab radioimmunoassay. However, there was still no correlation between cytotoxicity and Mi-ab/TPO-ab. Our data presented in this study clearly demonstrate that cytotoxicity in the microsomal antibody negative sera is mediated by an IgG. Pre-incubation of TPO-ab negative and positive sera with purified TPO had no influence on the cytotoxic effect in the majority of sera tested. Although we cannot explain the reason for the decrease of cytotoxic activity in 2 sera after pre-incubation with TPO, our data suggest that cytotoxic IgGs and TPO antibodies are induced by and directed against different antigen structures on the thyroid cell surface. In preliminary Western blot experiments with solubilized thyroid membrane preparations and immunoblotting of Mi-ab negative/cytotoxic positive sera, we could find no binding of the cytotoxic antibody to thyroid membrane fractions, which might be due to the lower sensitivity of the Western blot technique in comparison to the ADCC method. Furthermore, sera with high cytotoxic activity did not bind to the TPO antigen, which gives further evidence that cytotoxic antibodies are not identical with TPO antibodies. This finding supports the hypothesis that TPO antibodies are not cytotoxic and that the cytotoxic effect is mediated by an antibody not yet identified.

No simultaneous analysis has been published for a comparison between antibody-dependent complement-mediated and cell-mediated cytotoxicity. This is mainly due to different experimental procedures. In the complement-mediated cytotoxicity assay, thyroid cells lose their susceptibility to cytotoxic antibodies shortly after becoming attached to the wall of the cultures flasks and do not regain it after transfer in solution by trypsinization (18). In the ADCC assay, on the other hand, the thyroid cells are susceptible to cytotoxic antibodies even after several days of culture and after forming a monolayer. Optimal results for low nonspecific lysis, high percent specific lysis, and low intra-assay variance were obtained when the cells were cultured for 3 days (own observations). Although the expression of microsomal antigen is known to diminish in time under cell culture conditions, thyroid cells cultured for 3 days still retain microsomal/TPO antigen on their surface (19). Therefore, the absence of TPO antigen expression on the thyroid cell surface cannot explain the fact that we found no correlation between cell lysis and microsomal/TPO antibody titres. The question as to whether cytotoxic antibodies determined by complement- and cell-mediated lysis are identical remains unsolved. It is conceivable that different cytotoxic antibodies react with different effector components, in one case with the complement system and in another with effector cells. It is well known that IgG subclasses have a different affinity to cell-mediated cytotoxic reactions, e.g. IgG was shown to be ineffective in eliciting antibody-dependent cell-mediated cytotoxicity in contrast to the other IgG subclasses (20).

In conclusion, our data give evidence that, besides the well-known thyroid autoantibodies, a further antibody exists which is responsible for thyroid cell destruction in autoimmune thyroid disease. This antibody can occur either alone or together with the other autoantibodies and might, in some cases, be the only factor to prove the autoimmune pathogenesis of the disease. Further studies are in progress to support the hypothesis of the diversity of cytotoxic and thyroid peroxidase antibodies.

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