Antithyroperoxidase Antibody-Dependent Cytotoxicity in Autoimmune Thyroid Disease

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Context: Thyroid antibody-dependent cytotoxicity has been reported in autoimmune thyroid disease (AITD). Indeed, the role of thyroperoxidase (TPO) autoantibodies (aAbs) in complement-mediated damage by binding to TPO expressed on the surface of human thyroid cells was demonstrated, whereas their activity in antibody-dependent cell cytotoxicity (ADCC) is not well established.

Objective: The aim of this study was to define the partners involved in antibody and complement-dependent cytotoxicity (CDC) in AITD and characterize which effector cells are involved in cytotoxicity mediated by anti-TPO aAbs using a chromium release assay.

Results: The relative capability of anti-TPO aAbs to mediate ADCC using human thyroid cells in culture varies from 11 to 74.5%, depending on the effectors cells used. The human monocyte cell line HL60 gives a better lysis than the THP-1 cell line as effector cells. It seems obvious that the mechanism of ADCC is mediated quite exclusively by FcγRI. Indeed, the two effector cell lines differ by the level of the FcγRI expression (91.83% for HL-60 cells and 22.55%t for the THP-1). In addition to ADCC, the anti-TPO aAbs mediate the destruction of thyrocytes by CDC (56%).

Conclusions: These results demonstrate that anti-TPO aAbs can damage cultured thyroid cells by ADCC and CDC mechanisms. The monocytes, via their FcγRI, are important effector cells in ADCC mediated by anti-TPO aAbs and may contribute with T cells to the destruction of thyroid gland in AITD. **(*J Clin Endocrinol Metab* 93: 929–934, 2008)**

Autoimmune thyroid diseases (AITD) cover a large spectrum of disease from hyperthyroidism in Graves' disease (GD) to thyroid destruction in Hashimoto's thyroiditis. In both Graves' disease and Hashimoto's thyroiditis, autoantibodies (aAbs) specific of thyroid autoantigens, such as thyroglobulin (Tg), thyroperoxidase (TPO), and/or the TSH receptor, are present. Besides the role of AITD marker, these antibodies could be implicated in the pathogenic mechanisms of these diseases (1, 2). TSH receptor stimulating or blocking antibodies influence the action of TSH and may lead to a hyper- or a hypothyroidism (3). Whereas the role of the anti-TSH receptor antibodies in Grave's disease is admitted, the pathogenic role of anti-Tg and TPO aAbs is still discussed. Contrary to the TPO, Tg is not expressed on the surface of the thyrocyte and thus limits a role of anti-Tg aAbs in antibody-dependent cell cytotoxicity (ADCC). Moreover, anti-Tg antibodies do not fix the complement (4). All these data suggest that the antibodies that can mediate cytotoxic activity would be rather the anti-TPO antibodies.

Autoantibodies against TPO are present at high concentration in 90% of Hashimoto's thyroiditis (3, 5). They are involved in thyroid cell destruction through cytotoxic mechanisms mediated by effector cells and/or complement activation. Bogner *et al.* (6) are the first to show thyroid ADCC in Hashimoto's thyroiditis. Later, it is revealed that affinity-purified polyclonal TPO aAbs can mediate ADCC in porcine thyroid cells (7). Whereas Metcalf *et al.* (8) do not show correlation between IgG subclasses of TPO antibodies and lysis of thyroid cells in their *in vitro* assay, Guo *et al.* (9) show that TPO aAbs of IgG_1 subclass, and not

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Abbreviations: aAb, Autoantibody; ADCC, antibody-dependent cell cytotoxicity; AITD, autoimmune thyroid disease; CDC, complement-dependent cytotoxicity; D-PBS, Dulbecco's PBS; PBMC, peripheral blood mononuclear cell; Tg, thyroglobulin; TPO, thyroperoxidase.

IgG_4, mediate thyroid cell damage. In addition, TPO antibodies may produce complement-mediated cytotoxicity (CDC) *in vitro* by binding to TPO expressed on the thyroid cell surface (10, 11). It is also shown that some fractions of the complement are activated by the cell surface TPO to induce the cell lysis by CDC (12). All these findings support the hypothesis that aAbs directed against TPO are involved in the autoimmune destruction of thyrocyte.

However, the question of what effector cells can produce the cytotoxic effect is not addressed. In all the ADCC studies described above, the authors used peripheral blood mononuclear cells (PBMCs) as effector cells in cytotoxic assays. For many years, it is underlined that AITD are associated with mononuclear cell infiltration of the thyroid gland (13). Furthermore, the production of monocyte chemoattractant protein-1 by thyroid cells, a chemoattractant for monocytes, suggests a possible role of the thyrocytes in the recruitment of monocytes from the blood stream into thyroid stroma (14). Nevertheless, the involvement of monocytes in ADCC was not studied in AITD.

The aim of the present study was to confirm the pathogenic effects of anti-TPO aAbs in the destruction of thyroid cells. For this purpose, we set up of a cytotoxic test using primary cultures of human thyroid cells as target cells of anti-TPO aAbs. Two mechanisms of cytototoxicity were studied: ADCC and CDC. ADCC is first done using PBMCs as effector cells to confirm the ability of anti-TPO aAbs to mediate cell lysis. Then to investigate the type of effector cells involved in ADCC, we used monocyte cells lines (HL-60 and THP-1) as effector cells. For the first time, we show that human anti-TPO aAbs purified from patient sera with AITD are able to cause the destruction of the thyroid gland by ADCC when associated with monocytes. The biological effect is triggered by the FcγRI and FcγRII expressed on these cells.

Patients and Methods

Patients' sera and antibodies

Sera from patients suffering from Graves' disease were obtained from Dr. B. Guerrier (Gui de Chauliac Hospital, Montpellier, France) and Dr. L. Baldet (Lapeyronie University Hospital, Montpellier, France). Human IgG antibodies were purified on protein G column and the concentration determined by the A_{280nm}, $E^{0.1\%}$ of 1.40. A rabbit polyclonal antihuman TPO antibody was produced in our laboratory.

Primary human thyrocytes cultures: target cells

Tissues from patients suffering from Graves' disease were obtained from Dr. B. Guerrier (Gui de Chauliac Hospital, Montpellier, France). Each patient's tissue was used to prepare a single primary cell culture as previously described (15). Briefly, the tissue was minced, digested with collagenase (Sigma, St. Louis, MO) in Hank's balanced salt solution medium (PAA Laboratories, Linz, Austria) for 1 h at 37 C. The digested tissue was filtered through a sterile gauze, washed with Coon-modified Ham's F-12 medium (Sigma) containing 2.6 g/liter sodium carbonate (Sigma), 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mmol/liter glutamine (Life Technology, Paisley, UK) and supplemented with 5% fetal calf serum (PAA Laboratories). The cells were seeded in culture medium supplemented with bovine TSH (1 U/liter; Sigma) and the following five nutrients: 10 mg/ml human insulin; 10 μg/liter somatostatin; 6 μg/liter human transferrin; 10^{-8} M hydrocortisone; and 10 μg/liter glycyl-histidyl-lysine acetate to maintain the thyrocytes in a stimulating environment (16). The cells were incubated at 37 C in a humidified atmosphere containing 5% CO_2. After 24 h, the culture supernatant was removed by aspiration and fresh medium was added.

Immunofluorescence

Thyroid cells grown on LabTech chamber slide system (Sigma) were used at 75% confluence. Cells were washed with PBS (pH 7.4) containing $CaCl_2$ and $MgCl_2$ and fixed with 3% paraformaldehyde for 30 min and quenched with 50 mM NH_4Cl for 10 min. After two washings with PBS, the slides were saturated with 2% BSA-gelatin solution and then incubated with the polyclonal rabbit anti-TPO antibody (diluted 1:1000) overnight at 4 C. After three washings, the cells were incubated for 1 h with fluorescein isothiocyanate-conjugated antirabbit antibody (diluted 1:100; Vector Laboratories, Peterborough, UK). After three additional washings, the cells were covered with citifluor (Citifluor, London, UK) and observed with an upright fluorescence microscope using the facilities of RIO imagery platform (Montpellier, France). The negative control was performed using only the conjugated antibody.

Effector cells

Peripheral mononuclear cells obtained from healthy donors (Etablissement Français du Sang, Montpellier, France) were separated from blood by Ficoll density gradient (Histopaque-1077; Sigma) and resuspended in RPMI 1640 (Cambrex, Verviers, Belgium) supplemented with fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mmol/liter glutamine. PBMCs from only one donor were used.

HL-60 and THP-1 cell lines, human monocyte lines (provided by J. Dornand, Montpellier, France) were grown in the same medium as PBMCs. Five days before the experiment, 10^{-7} M of 1,25-dihydroxyvitamin D_3 (vitamin D_3) was added to induce the differentiation of HL-60 cells in monocyte.

Flow cytometric analysis was used to study the FcγR expression on effector cells and the antibody binding with these receptors

Effector cells were rinsed and pelleted (5 min, 1000 rpm, 4 C) in Dulbecco's PBS (D-PBS) (Cambrex) containing 2% FCS (F-buffer). The cells (~10^6) were incubated with 200 μl of F-buffer containing 10 μg/ml of human anti-Fc γ receptor antibodies (CD16, 32 and 64, respectively, FcγRIII, II, and I (PharMingen, Becton Dickinson, Franklin Lakes, NJ). To study the antibody binding on Fcγ receptor, 10 μg/ml of human anti-TPO aAbs was preliminarily incubated with TPO antigen for 45 min at 4 C before being incubated with cells (~10^6) for 90 min at 4 C. In both cases, the cells were washed twice and then incubated in 200 μl of F-buffer with 10 μg/ml of fluorescein-conjugated antihuman IgGγ chain-specific antibody (Sigma) for 60 min at 4 C in the dark. As negative control, cells were incubated with only the conjugated antibody. After washings with D-PBS, the cells were analyzed (10,000 events) with a cytofluorometer (FACScan; Becton Dickinson).

ADCC assay

The effector cells, used in ADCC assays, were PBMCs, HL-60, and THP-1. Cytotoxic assays were performed according to previously described protocols (7, 9). Target cells (Graves' thyroid cells) were removed from the culture flasks using HEPES-EDTA buffer [HEPES 10 mM, EDTA 3 mg/ml (pH 7.0)] and washed in PBS. Aliquots containing 10^6 cells were incubated (37 C, 60 min, 5% CO_2) in culture medium with 100 μCi ^{51}Cr. After washings to eliminate ^{51}Cr, which was not incorporated, labeled target cells were incubated (4 C, 45 min) with anti-TPO antibodies purified from patients' sera (50 μg/ml), with irrelevant human IgG1 or culture medium alone as control. After the incubation, the cells were distributed (2×10^4 cells/well) in 96-well U-bottomed culture plates. Tests and control were performed in triplicate. Target cells were incubated with effector cells (effector to target ratio, 50:1 PBMCs, 12:1 cell line). The target cells were used to determine the spontaneous ^{51}Cr release with culture medium only and the total ^{51}Cr release by the ad-

dition HCl, 1 N. The culture plates were incubated for 6 h (37 C, 5% CO_2). After centrifugation (5 min, 1000 rpm), aliquots (100 μl) of the supernatant were removed for γ-counting. Cytotoxicity was expressed as specific 51 Cr release calculated as follows: lysis = [(counts per minute with effector cells) − (counts per minute with culture medium)]/[(counts per minute with HCl) − (counts per minute with culture medium)].

Flow cytometric analysis of the binding of the C1q complement component

Human thyroid cells were removed from the culture flasks using HEPES-EDTA buffer, rinsed, and pelleted (5 min, 1000 rpm, 4 C) in F-buffer. The cells ($\sim 10^6$) were incubated with 200 μl of F-buffer containing 10 μg/ml of human anti-TPO aAbs for 90 min at 4 C. The cells were washed twice and then incubated in 200 μl of F-buffer with human C1q complement component for 60 min at 4 C. After two washings, 10 μg/ml of fluorescein-conjugated antihuman C1q complement component antibody (Sigma) was added for 45 min at 4 C in the dark. As negative control, cells nonopsonized by human anti-TPO aAbs were incubated with C1q complement component and the conjugated antibody or with only the conjugated antibody. After washings with D-PBS, the cells were analyzed (10,000 events) with a cytofluorometer (FACScan; Becton Dickinson).

CDC assay

To test complement-mediated cytotoxicity, the target cells were radiolabeled with ^{51}Cr for 60 min at 37 C as for the ADCC protocol. After washing, labeled target cells were distributed (2×10^4 cells/well) in 96-well U-bottomed culture plates. Target cells (triplicate wells) were incubated (4 C, 45 min) in culture medium alone or with human anti-TPO antibodies (50 μg/ml). Cultures were incubated for 4 h at 37 C with guinea-pig serum (Sigma). After centrifugation (5 min, 1000 rpm), aliquots (100 μl) of the supernatant were removed for γ-counting. Cytotoxicity was expressed as specific ^{51}Cr release calculated according to the following formula: lysis = [(counts per minute with antibodies and complement) − (counts per minute with complement alone)]/[(counts per minute with HCl, 1 N) − (counts per minute with complement alone)].

Results

Primary thyroid cell cultures express TPO

To investigate the cytotoxic susceptibility of human thyroid cells to ADCC in presence of effector cells and anti-TPO antibodies from patients with AITD, we need to characterize the cell partners of the ADCC: human thyroid cells and effector cells. First, we confirm the expression of TPO on human thyroid cells in primary culture by immunofluorescence studies using a rabbit polyclonal antihuman TPO antibody previously obtained in our laboratory. As shown in Fig. 1A, this experiment clearly shows that TPO is expressed at the membrane surface and in the cytoplasm of human thyroid cells in primary culture, as a punctuated staining. In addition, flow cytometric analysis were performed to test the capacity of patients' sera anti-TPO aAbs to bind human thyroid cells. As indicated in Fig. 1B, flow cytometric pattern shows a strong binding of patients' sera anti-TPO aAbs. Thus, we confirm the expression of TPO on primary culture of thyroid cells and demonstrate the ability of patients' sera anti-TPO aAbs to bind such thyroid cells.

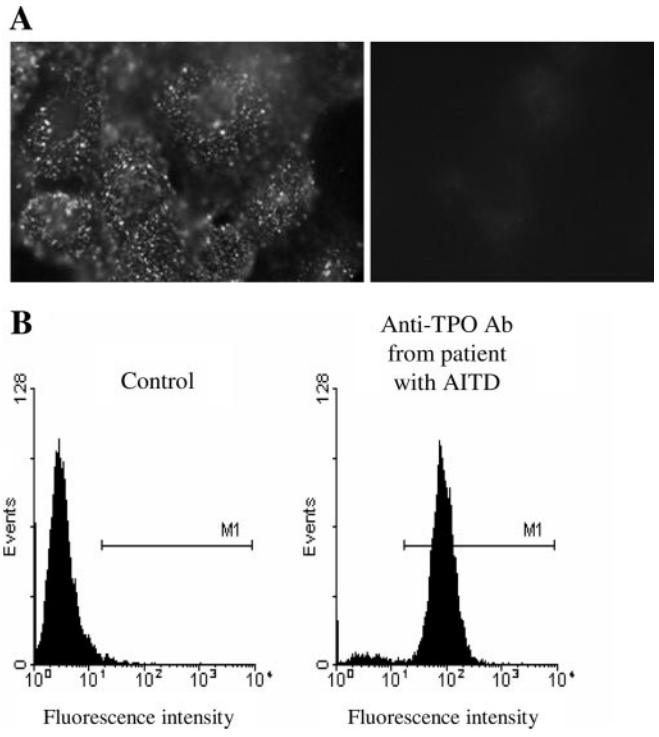

FIG.1. Human thyroid cell TPO expression. A, Immunofluorescence staining of thyroid cells by fluorescein isothiocyanate-rabbit anti-TPO antibody (*left panel*) and fluorescein isothiocyanate-rabbit antibody as control (*right panel*). B, Flow cytometric analysis of the binding of human anti-TPO aAbs from patients' sera with AITD to thyroid cells.

Effector cell characteristics

To identify whether monocytes could be involved as effector cells for anti-TPO aAbs mediated ADCC against human thyroid cells, we chose two monocyte cell lines (HL-60 and THP-1 lines). These cells were characterized in term of monocytes surface markers such as CD11b, CD14, and FCγR expression. The differentiation of HL-60 in monocyte with vitamin D_3 was underlined by the increased expression of CD11b (by 61.45–91.48%), whereas more than 90% of THP-1 cells naturally express

TABLE 1. Expression of FcγR (CD16, CD32, CD64) on human monocytic cell lines and PBMCs (lymphocytes, monocytes, and granulocytes)

		CD11b	CD14	CD16	CD32	CD64
HL-60	No stimulation	61.45		0.24	98.37	15.95
	Vitamin D_3 stimulation	91.48	98.27	0.06	93.24	91.83
THP-1		92.01	56.40	0.32	96.79	22.55
	Lymphocytes			87.91		
PBMCs	Monocytes		90.92		88.12	12.00
	Granulocytes		62.13		63.95	4.49

Values represent percentage of fluorescence. The results, presented here, correspond to the average of at least two experiments.

CD11b. Using anti-CD14 antibody, 56.40% of THP-1, 98.26% of HL-60, and 90.92% of monocyte population of PBMCs are stained as shown in Table 1.

FcγRII and I (CD32 and CD64) are expressed on HL-60 and THP-1 cell lines and monocytes present in PBMCs; indeed, FcγRIII is present only on the lymphocyte population of PBMCs. Interestingly, whereas the effector cells HL-60, THP-1, and PBMCs (monocytes) express FcγRII in the same proportion (93.24–98.37%), more than 91% of HL-60 cells but only 12% of PBMCs (monocytes) and 22.55% of THP-1 are stained with anti-FcγRI antibody (Table 1).

TPO aAbs binding on effector cells

Flow cytometric analysis was performed to compare the binding of human anti-TPO aAbs by their Fc region on HL60, THP-1, and PBMCs. As indicated in Fig. 2, similar cytometry patterns, showing the binding of the immune complex (TPO/anti-TPO aAbs) were obtained for the two monocytes cells lines. However, a higher staining was observed for HL60 (82.55%). For the cell populations present in PBMCs, 83.54% of the monocyte, 92.37% of the lymphocyte, and 94.97% of the neutrophile were stained by human anti-TPO aAbs. The use of anti-FcγR Abs abolish the immune complex binding on the effector cells and confirm the binding of human anti-TPO aAbs by their Fc region (data not shown).

TPO aAbs mediated thyroid ADCC

To evaluate the involvement of monocytes as effector cells in TPO aAb-mediated ADCC, cytotoxic assays were performed using human thyroid cells obtained from patients undergoing surgery for Graves' disease as target cells and PBMC and monocyte cell lines (HL-60 and THP-1) previously described as effector cells (Fig. 3). The use of PBMCs, which include T cells, B cells, monocytes, neutrophile, and natural killer cells, gave 11% of ADCC. Interestingly, human anti-TPO aAb-mediated Cr^{51} release was higher using HL-60 than THP-1 cell line (mean, 74.5 *vs.* 18.8%). These results corroborate FcγR expression. Indeed, monocyte HL-60 and THP-1 differ for their expression of FcγR, suggesting that ADCC could pass preferentially by the binding of the anti-TPO aAbs on FcγRI expressed by these effector cells.

Anti-TPO aAbs bind C1q component and have CDC effect

The complement activation via the classical pathway is initiated by the binding of an immune complex (cell expressing TPO/anti-TPO aAbs) with the C1 component. It follows an enzymatic cascade, which ends to the formation of the membrane attack complex (C5b-9) and leads to the lysis of the target cell. We investigated, by flow cytometry, whether C1q component can bind on the immune complex cell expressing TPO/anti-TPO aAbs. As expected, the results given in Fig. 4 show that C1q was able to bind anti-TPO aAb complexes at the surface of human thyroid cells.

The role of complement was investigated. In Fig. 5, 60% of specific Cr^{51} release was observed when Cr^{51}-labeled human thyroid cells were added with human anti-TPO aAbs and guinea pig serum. Negative controls were done by incubation of the target cells with serum alone or with an irrelevant IgG.

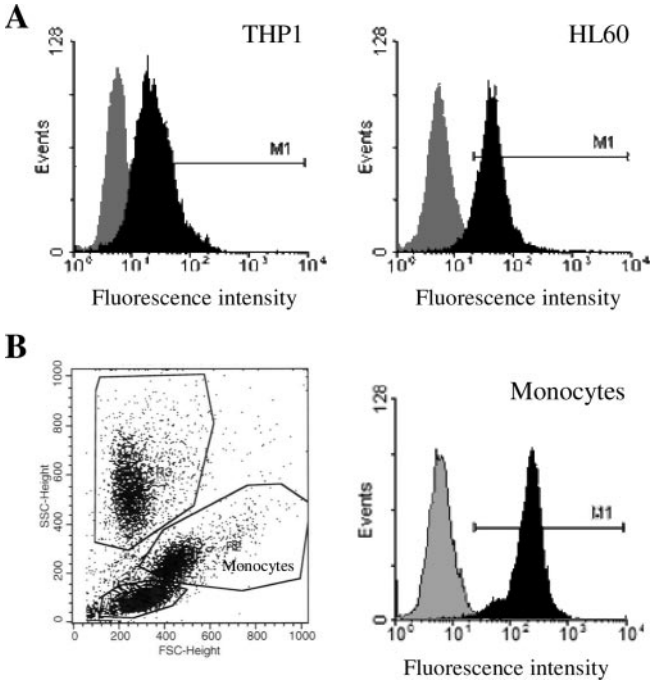

FIG. 2. Flow cytometric analysis of the binding of purified anti-TPO aAbs on human monocytic cells and PBMCs. A, Binding on monocyte cell lines (THP-1 and HL-60). B, Binding on monocytes from PBMCs. *Gray areas* represent cells incubated with fluorescein isothiocyanate-conjugated antibodies. *Black areas* represent cells incubated with human anti-TPO antibodies from patient with AITD and fluorescein isothiocyanate-conjugated antibodies.

Discussion

ADCC, a cytotoxic mechanism, depends on complex interactions between target cells, antibodies, and effector cells. Even if thyroid-specific ADCC has been described in AITD and human TPO aAbs have been demonstrated as implicated in cytotoxic mechanism by several group (6–9, 17), the mechanisms involved

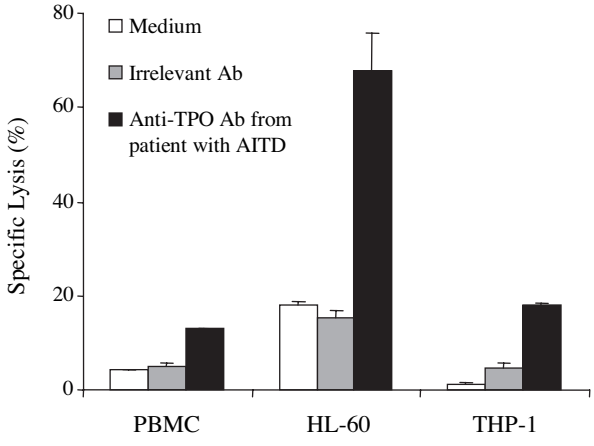

FIG. 3. ADCC assay. Human thyroid cells were incubated with culture medium alone (□), irrelevant antibodies (Ab; ▨), or human anti-TPO aAbs purified from patients' sera with AITD (■) with different effector cells (PBMCs, HL-60, THP-1). Values are the percentage of specific lysis (mean ± SD).

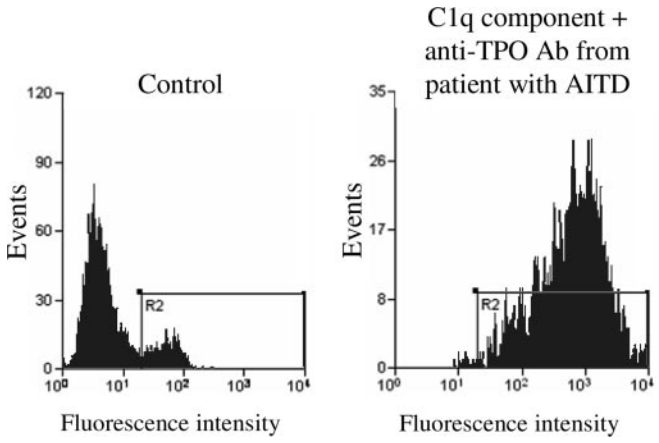

FIG. 4. Flow cytometric analysis of C1q complement component binding on immune complexes formed by human anti-TPO aAbs from patients' sera with AITD and thyroid cells expressing TPO.

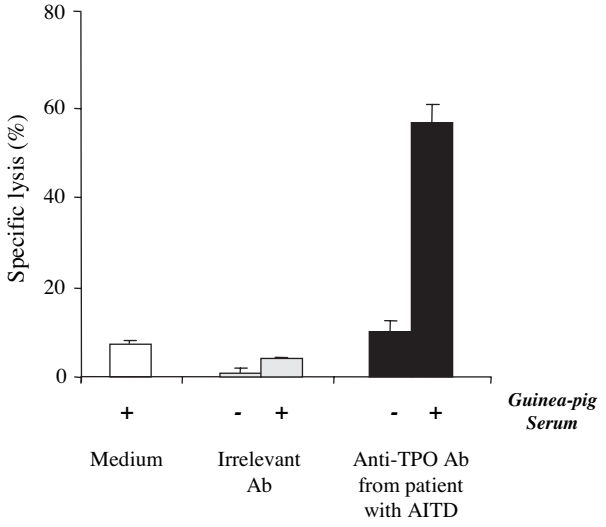

FIG. 5. CDC assay. Human thyroid cells expressing TPO were incubated with culture medium alone (□), irrelevant antibodies (Ab; ▨), or human anti-TPO aAbs purified from patients' sera with AITD (■) with (+) or without (−) guinea pig serum. Values are the percentage of specific lysis (mean ± SD).

in pathological activities of anti-TPO aAbs are still largely unknown. Herein we demonstrate that anti-TPO aAbs present in patients' sera suffering from AITD can mediate cytotoxic activities by ADCC and CDC of human thyroid cells and that monocytes are potential effector cells through their FcγRI.

The efficiency of ADCC can be modulated by antibody isotype (9), immunoglobulin glycosylation level (18–21), the type of effector cells (9), cytokine pattern (22, 23), or FCγR involved in the binding (21, 24).

Concerning the antibody isotype, the Fc region, which differs in immunoglobulin molecules of different classes and subclasses, is responsible of the biological effects of the antibody. Indeed, Guo *et al.* (9) demonstrated that human TPO-specific Fab IgG_1 mediates cytotoxic effects of human thyroid cells *in vitro*, whereas the same Fab converted in IgG_4 does not. Because in patients with AITD, anti-TPO aAbs are mainly IgG_1 and IgG_4 (25, 26) and subclass difference is pertinent to the ability of thyroid aAbs to mediate ADCC, we used, in our experiments, purified human anti-TPO aAbs from patients' sera in which IgG_1 subclass is present in higher proportion (data not shown). Nonetheless, the efficiency of ADCC is variable in function of effector cells used. These results demonstrate that others factors are crucial for the efficiency of cytotoxicity.

The functional activities of human anti-TPO aAbs can be assessed by their ability to bind to effector cells via FcγR. The presence of FcγR on the cells surface is known and natural killer cells express FcγRIII (or CD16), whereas neutrophile and monocytes express FcγRII and I (CD32 and 64, respectively). Flow cytometry experiments show strong binding of anti-TPO complexes for monocyte cell lines, particularly HL-60. This differential binding of purified human anti-TPO aAbs from patients' sera to monocytes cell lines HL60, THP-1 and PBMCs is consistent with preferential IgG1 antibody binding to Fc receptors (27, 28). ADCC is mediated by FcγRI, -RII, and -RIII expressed on effector cells. Nevertheless, FcγRI has a higher affinity for IgG than do FcγRII and FcγRIII. Because FcγRI is greatly expressed on HL-60, it seems likely that the binding of anti-TPO aAbs is higher on HL60 than THP-1 and PBMCs. The FcγRI and FcγRII exist on the monocyte cell surface. The ADCC could be the result of interaction of the immune complex with the FcγRI and/or FcγRII. These effector cell lines expressed the FcγRII in the same proportion than do the THP-cells but differed for the expression of the FcγRI. Because ADCC is correlated with the expression of FcγRI, it seems obvious that FcγRI expression is crucial for cytotoxic activity.

On the other end, the CD11b, a member of the β2 integrin family, expressed on monocytes could be implicated for FcγR mediated cytotoxicity. Several studies point to possible physical interaction between Mac-1 and FcRs during ADCC (29, 30). In our study, CD11b is expressed as well as on THP-1 than HL60 and in an equivalent proportion, suggesting that CD11b in not involved in the differences observed for ADCC mediated by the two monocyte cell lines.

Finally, a prominent feature of autoimmune thyroid disease is infiltration of the thyroid gland with lymphocytes and monocytes/macrophages (31). The importance of tissue-infiltrating immune cells for the development and perpetuation of autoimmune thyroid disease is indisputable, but the involvement of monocytes in ADCC was not explored. We observed in thyroid tissue from Graves' disease patients, by immunofluorescence studies, the presence of CD14+ cells infiltrating the thyroid (data not shown). In the present study, we show that anti-TPO aAbs mediate efficacious ADCC using monocyte cell line HL-60. Interestingly, it appears that in this cytotoxic process, monocytes, via their FcγRI, engage and kill thyroid target cells coated with anti-TPO aAbs.

The CDC mechanism mediated by anti-TPO aAbs and heterologous complement can potentialize the destruction of the target cells. It may be interesting to explore the respective involvement of ADCC and CDC in human thyroid disease.

TPO, one of the main thyroid aut-antigens, is expressed on the thyroid cell surface as well as in the cytoplasm. Because TPO aAbs are present in patients' sera suffering from an AITD, and it is reasonable to think that these aAbs are not responsible for the initial damage of the gland but could contribute to thyroid de-

struction once follicular structure has been disrupted in Hashimoto thyroiditis. As opposed to being simple markers of thyroid damage, TPO aAbs may play a role in the induction of thyroid dysfunction *in vivo* and in the amplification and progression of the disease by cytotoxic mechanisms.

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