Recombinant Thyroid Peroxidase-Specific Fab Converted to Immunoglobulin G (IgG) Molecules: Evidence for Thyroid Cell Damage by IgG1, but Not IgG4, Autoantibodies*

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ABSTRACT
A recombinant autoantibody Fab (SP1.4) to thyroid peroxidase (TPO), cloned from intrathyroidal B cell immunoglobulin genes, interacts with an epitope on TPO recognized by all patients with autoimmune thyroid disease. To compare the biological properties of IgG1 and IgG4 TPO autoantibodies, we converted Fab SP1.4 to full-length immunoglobulins. The SP1.4 heavy and κ light chain variable region genes, spliced by overlap PCR to a mammalian signal peptide, were transferred to expression vectors for human IgG1, IgG4, and κ L chains. Plasmids containing the IgG1 (or IgG4) heavy chain and the κ L chain were cotransfected into SP2/0 mouse myeloma cells. Cells secreting TPO autoantibodies were cloned, and IgG1-SP and IgG4-SP were affinity purified from medium using protein G. Their subclass specificities were confirmed by enzyme-linked immunosorbent assay and fluorometry after binding to Chinese hamster ovary cells expressing cell surface TPO. Further confirmation of SP1.4 Fab conversion to full-length molecules was the ability of protein A to precipitate IgG1-SP and IgG4-SP complexes to [125I]TPO. IgG1-SP1.4, IgG4-SP1.4, and Fab SP1.4 had similar high affinities for TPO (Kd = ~2 × 10⁻¹⁰ mol/L). Complexes of [125I]TPO and IgG1-SP (but not IgG4-SP) bound to peripheral blood mononuclear cells (PBMC), but not to a B cell line. Flow cytometry demonstrated Fc receptors FcγRI, FcγRIII, and FcγRIII on PBMC, but only FcγRII on the B cell line. Together, these data indicate that IgG1-SP/TPO complexes bind to either FcγRII on monocytes or RII on natural killer cells. In assays for antibody-dependent cytotoxicity using PBMC, ⁵¹Cr release was higher for thyroid cells preincubated with IgG1-SP (13.4%) than with IgG4-SP (2.5%) or with culture medium alone (~0.7%). No specific ⁵¹Cr release was observed when either fibroblasts or Chinese hamster ovary cells expressing cell surface TPO were used as target cells.

In conclusion, a human TPO-specific Fab converted to IgG1, but not IgG4, can mediate cytotoxic effects on human thyroid cells in vitro. These observations support the clinical relevance of TPO autoantibody subclass distribution and emphasize the likelihood that, as opposed to being simple markers of thyroid damage, TPO autoantibodies may play a role in the induction of thyroid dysfunction in vivo. (J Clin Endocrinol Metab 82: 925–931, 1997)

CYTOTOXICITY of thyroid autoantibodies was first suspected when autoantibodies to an unknown thyroid antigen in Hashimoto sera were observed to fix complement (1). Previously identified thyroglobulin autoantibodies did not have this function (2). Subsequently, both complement fixation and a serum factor capable of thyrocyte cytotoxicity were found to be associated with autoantibodies to thyroid microsomes (3–5). The thyroid microsomal antigen is now known to be thyroid peroxidase (reviewed in Ref. 6). More recent studies have supported a role for microsomal/thyroid peroxidase (TPO) autoantibodies in complement-mediated damage by binding to TPO expressed on the surface of human thyroid cells (7–9). However, complement inhibitors are expressed on human thyroid cells in vivo (10).

Besides complement-induced cytotoxicity, damage can also be mediated by an antibody-dependent cell cytotoxic mechanism (ADCC). In this process, effector cells (macrophages or natural killer cells), via their Fcγ receptors (FcγR) engage and kill target cells coated with antibody. Indeed, thyroid autoantibodies can damage cultured thyroid cells by ADCC (11). However, unlike complement-mediated thyroid autoantibody damage, the autoantigen(s) involved in ADCC is not well established. Thus, an association between thyroid microsomal/TPO autoantibodies and ADCC has been found in some studies (11–13), but not in others (14–16).

As is well known, antibodies are bifunctional molecules. The Fab region binds to antigen. The Fc region, which differs in immunoglobulin molecules of different classes and subclasses, is responsible for the biological effects of the antibody (reviewed in Ref. 17). For example, IgG1 and IgG3 antibodies to red blood cells are more efficient than IgG2 or IgG4 antibodies of the same specificity in lysing erythrocytes by ADCC (18). This subclass difference is pertinent to the ability of thyroid autoantibodies to mediate ADCC, because TPO autoantibodies are predominantly IgG1 and IgG4, with IgG2 in some patients and little if any IgG3 (19, 20). Based on the red blood cell model, it is possible that ADCC would be more effective with IgG1, rather than IgG4, TPO autoantibodies in patients’ sera.

We have previously isolated a panel of human monoclonal
TPO autoantibodies, expressed as Fab, by cloning immunoglobulin genes from patients’ intrathyroidal B cells (reviewed in Ref. 21). One of these Fab, SP1.4, interacts with a TPO epitope recognized by the sera of all patients with autoimmune thyroid disease (22). In the present study, we have converted Fab SP1.4 to full-length IgG molecules of two different subclasses, IgG1 and IgG4. Data obtained with these two antibodies, with identical antigen-binding regions, support the likelihood that TPO autoantibodies of subclass IgG1 mediate thyroid cell damage in vivo.

Materials and Methods

Plasmids for mammalian cell expression of TPO-specific IgG1 and IgG4

Construction of plasmids pAH4604-SP-G1 and pAN4621-SP-K for the expression of the TPO autoantibody SP1.4 full-length IgG1 heavy (H) chain and κ light (L) chain have been described previously (23) (Fig. 1). In brief, because the SP1.4 H and L chains in the bacteriophage λ vector (Immunozap, Stratagene, La Jolla, CA) contained bacterial signal peptides, in pAH4604-SP-G1 and pAN4621-SP-K, these signal peptides were replaced with the human TSH receptor signal peptide (24). The full-length IgG4 version of the SP1.4 H chain was generated by inserting the EcoRV/NheI fragment containing the VH, D, and J regions of SP1.4 from pAH4604-SP-G1 into the same sites in the H chain mammalian cell vector with human IgG4 constant regions (pAH4801; kindly provided by Dr. Sherie Morrison, University of California-Los Angeles). The new SP1.4 IgG4 H chain plasmid was called pAH4801-SP-G4 (Fig. 1).

Expression of IgG1 and IgG4 versions of Fab SP1.4

Plasmids pAN4621-SP-K (2 μg) and either pAH-SP-G1 or pAH-SP-G4 (20 μg), linearized with PvuI, were stably transfected into SP2/0 cells by electroporation and neomycin (G418) selection, as previously described in detail (23). Screening of 96-well plates for TPO antibody production was performed by enzyme-linked immunosorbent assay (ELISA) or [125I]TPO binding (see below). Cells from positive wells were cloned by limiting dilution. Two clones (IgG1-SP and IgG4-SP) that secreted high levels of IgG1 and IgG4 class TPO autoantibodies, respectively, were expanded to provide about 1 L medium. IgG was affinity purified using a protein G column (Pharmacia Biotech, Piscataway, NJ).

ELISA for IgG1-SP and IgG4-SP

TPO secreted into culture medium from Chinese hamster ovary (CHO) cells (25) was used to coat ELISA plates, as previously described (22). TPO autoantibodies IgG1-SP and IgG4-SP were detected using

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**Fig. 1.** Plasmids for expression of Fab SP1.4 VH and VL genes as full-length IgG1 and IgG4 molecules. A, IgG1 H chain vector (pAH4604-SP-G1) was constructed as previously described (23) by splicing the TSH receptor signal peptide to the SP1.4 VH-D-JH regions and ligation into pAH4604 (41). The IgG4 H chain plasmid (pAH4801-SP-G4) was derived by transferring the EcoRV/NheI fragment containing the SP1.4 VH-D-JH from pAH4604-SP-G1 into the IgG4 expression vector pAH4801. B, κ L chain plasmid pAN4621-SP-K. Construction of this plasmid has been described previously (23).
murine monoclonal antibodies to human IgG1, IgG4, or κ chains (NL16, RJ4, and QE11, respectively; Recognition Sciences, Birmingham, UK) and a horseradish peroxidase detection system (22).

**TPO autoantibody binding to CHO cells expressing TPO (CHO-TPO cells)**

IgG1-SP or IgG4-SP binding to CHO-TPO cells (25) was performed as previously described (26). After incubation in IgG1-SP or IgG4-SP, cells were exposed to biotin-conjugated, mouse monoclonal anti-human IgG1 or anti-IgG4 (Caltag, San Francisco, CA) and subsequently incubated with streptavidin-R-phycocerythrin (Caltag). Fluorescence was determined using a Perkin-Elmer fluorimeter (Norwalk, CT; emission, 575 nm; excitation, 488 nm).

**Protein A precipitation of [125I]TPO binding by IgG1-SP or IgG4-SP**

The assay was performed on duplicate aliquots using [125I]-labeled recombinant TPO, as previously described in detail (22). Background binding (~5% of the total counts), determined using normal human serum diluted 1:20, was subtracted from the values for IgG1-SP or IgG4-SP when calculating the percentage of [125I]TPO bound. Data obtained in the absence of increasing concentrations of unlabeled TPO were used to determine affinities by Scatchard analysis (27). In some assays, precipitations were performed using monoclonal anti-human κ chains (QE11, Recognition Sciences), as previously described for Fab SP1.4 (22).

**Binding of lymphoid cell populations of [125I]TPO/IgG1-SP or IgG4-SP complexes**

Peripheral blood mononuclear cells (PBMC) from a normal donor were separated by density centrifugation (Histopaque-1077, Sigma Chemical Co., St. Louis, MO). Before use in binding studies (see below), PBMC were incubated for 1 h at 37°C in petri dishes to elute IgG1/antigen complexes bound or free to monocytes/macrophages. Lymphoblastoid B cell lines (EBVL) were obtained by transformation of Graves’ lymphocytes with Epstein-Barr virus as previously described (23). FcyR expression was analyzed by flow cytometry. Aliquots of PBMC and EBVL (~10⁶ cells) were incubated (1 h, 4°C) with fluorescein isothiocyanate (FITC)-conjugated murine monoclonal antibodies as follows: FcyRI, anti-CD64 (clone 10.1); FcyRII, anti-CD32 (clone 2003); and FcyRIII, anti-CD16 (clone 3GS; all from PharMingen, San Diego, CA). Controls included unaltered cells and FITC-conjugated isotype controls (IgG1 and IgG2b; PharMingen). Cells were analyzed (10,000 events) using the Becton Dickinson FACScan-CELLQuest system (Mountain View, CA).

**Binding of [125I]TPO complexed with IgG1- or IgG4-SP to PBMC or EBVL was performed as follows. Aliquots (400 μL) of culture medium containing IgG1-SP or IgG4-SP were incubated overnight at 4°C with [125I]TPO (200,000 cpm; ~5 ng) in the absence or presence of excess unlabeled TPO (1 μg). As controls, radiolabeled TPO was incubated in aliquots of fresh culture medium with or without unlabeled TPO. The following day, PBMC or EBVL (dilute aliquots, 5 × 10⁶ cells) were resuspended in the antibody-antigen complexes. After incubation for 2 h at 4°C, the cells were washed twice with 3 mL buffer B (phosphate-buffered saline and 2% BSA), and [125I]TPO bound to the pelleted cells was determined by γ-counting.

**ADCC**

Effector cells in ADCC assays were PBMC from a normal donor (see above). As targets, we used 1) Graves’ thyroid cells (28), 2) CHO-TPO cells (25), and 3) human fibroblasts of thyroid tissue origin. To restore TPO expression by thyroid cell primary cultures, cells were cultured for 3–4 days with TSH (10⁻⁷ mol/L) (29). Cytotoxicity assays were performed according to previously described protocols (11, 13, 30). Target cells were detached from culture dishes by light trypsinization, and aliquots (~3 × 10⁵ cells) were incubated (4°C, 30 min) in RPMI 1640 with 10% FCS and IgG1-SP or IgG4-SP or (as a control) in culture medium alone. Subsequently, 75 μCi ⁵¹Cr (sodium chromate, DuPont, Boston, MA) was added, and incubation was continued (45 min, 37°C, 5% CO₂).

After washing twice with culture medium, labeled target cells were distributed (~3 × 10⁵ cells/well) in 96-well U-bottomed culture plates. Target cells (triplicate wells) were incubated with effector cells (PBMC: ~1.5 × 10⁵ cells/well; effector/target ratio, ~40:1). Triplicate aliquots of the same target cells were used to determine ⁵¹Cr release without PBMC and total ⁵¹Cr release by the addition of culture medium or detergent (5% Triton X-100; Sigma), respectively. The culture plates were centrifuged (2 min, 53 × g) and incubated for 18 h (37°C, 5% CO₂). After centrifugation (5 min, 550 × g), aliquots (100 μL) of the supernatant were removed for γ-counting. Cytotoxicity was expressed as specific ⁵¹Cr release (mean ± SD of triplicate wells) calculated as follows: specific ⁵¹Cr release = ([cpm with PBMC] – [cpm without PBMC])/total cpm released. Differences in specific ⁵¹Cr release were analyzed by Student’s t test.

**Results**

IgG subclass of recombinant TPO autoantibodies secreted by mammalian cells

SP2/0 mouse myeloma cells cotransfected with pAN4621-SK (SP1.4 κ L chain) and pAH4604-SG1 (SP1.4 IgG1 H chain; Fig. 1) secreted TPO antibody detectable by ELISA with anti-human IgG1, but not with anti-human IgG4 (Fig. 2, left panel). Conversely, SP2/0 cells cotransfected with the same L chain plasmid and pAH4801-SG4 containing the SP1.4 IgG4 H chain gene (Fig. 1) produced TPO antibody detectable with anti-IgG4, but not anti-IgG1 (Fig. 2, right panel). Both IgG1-SP and IgG4-SP could also be detected with monoclonal anti-human κ. The subclass specificity demonstrated by ELISA using soluble TPO was confirmed by fluorometry (Fig. 3) using full-length TPO expressed on the surface of CHO cells (25).

**Protein A precipitation of [125I]TPO bound by IgG1-SP and IgG4-SP**

Further confirmation of the conversion of the SP1.4 Fab into full-length IgG1 and IgG4 was the ability of protein A to precipitate IgG1-SP and IgG4-SP complexed to [125I]TPO. Competition for this binding by increasing amounts of un-
labeled TPO (Fig. 4A) permitted Scatchard analysis (27) of the IgG1-SP and IgG4-SP affinities for TPO. The affinities ($K_d$) of $1.7$ and $1.9 \times 10^{-10}$ mol/L for IgG1-SP and IgG4-SP, respectively (Fig. 4, B and C), were essentially identical to that previously observed for Fab SP1.4 ($2.2 \times 10^{-10}$ mol/L) (22).

Relative concentrations of IgG1-SP and IgG4-SP

The above experiments were performed using IgG affinity purified with protein G from medium containing FCS. Despite its very low concentration in this medium, some bovine IgG was copurified with the TPO autoantibodies. Consequently, we could not determine the absolute concentrations of IgG1-SP and IgG4-SP. By ELISA, using antihuman $k$, the activity of IgG1-SP was approximately 10-fold greater than that of IgG4-SP (Fig. 2). Similarly, 10-fold more IgG4-SP than IgG1-SP (1:30 vs. 1:300 dilution) was required to attain comparable (23.9% vs. 23.8%) [125I]TPO binding as detected by precipitation with the same anti-$k$ antibody. Thus, both methods provided similar estimates of the relative functional concentrations of the two autoantibodies. Therefore, for all functional assays (below), IgG4-SP and IgG1-SP were used at a 10:1 ratio.

FcγR binding of recombinant IgG1-SP and IgG4-SP

The functional activities of the IgG1 and IgG4 Fc regions introduced into the SP1.4 Fab can be assessed by their ability to bind to lymphoid cells via FcγR. PBMC, which include T cells, B cells, monocytes, and natural killer cells, bound higher levels of [125I]TPO/IgG1-SP than [125I]TPO/IgG4-SP immune complexes (Fig. 5A). Inclusion of excess unlabelled TPO during complex formation reduced radiolabel binding by PBMC to background levels, namely culture medium without TPO autoantibody. In contrast, no specific binding to Epstein-Barr virus-transformed B cells (EBVL) could be detected (Fig. 5B). Flow cytometry of PBMC revealed the presence of all three forms of FcγR, namely FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16), on the surface of some cells in the population (Fig. 6, left panels). In contrast, EBVL had only FcγRII (CD32) on their surface (Fig. 6, right panels). Taken together, the data in Figs. 5 and 6 indicate that IgG1-SP/TPO complexes bind to lymphoid cells via either their FcγRI or FcγRIII.

ADCC

We examined the relative abilities of IgG1-SP and IgG4-SP to mediate ADCC using human thyroid cells in primary culture. In three separate experiments, IgG1-SP-mediated 51Cr release (mean, 13.4%) was higher than that for IgG4-SP (mean, 2.5%) as well as that for medium without antibody (mean, 0.7%; Fig. 7, upper panel). The significance of the difference between IgG1-SP and IgG4-SP in the individual experiments (each with triplicate values) was $P < 0.001$, $P < 0.001$, and $P = 0.05$, respectively. No specific 51Cr release was observed when either fibroblasts or CHO-TPO were used as target cells (Fig. 7, lower panel). Indeed, PBMC reduced the spontaneous release of 51Cr in these cells regardless of the presence or absence of IgG1-SP or IgG4-SP.

**Fig. 3.** Fluorometric analysis of IgG1-SP and IgG4-SP binding to CHO-TPO cells. Binding was assessed using biotinylated anti-IgG1 (anti-IgG1-bio) and anti-IgG4 (anti-IgG4-bio) and subsequent development of a signal with streptavidin conjugated to phycoerythrin (Sa-PE). Fluorescence is expressed in arbitrary units.

**Fig. 4.** Binding affinities of IgG1-SP and IgG4-SP for TPO measured by protein A precipitation. A, Radiolabeled TPO binding in the presence of increasing concentrations of unlabeled TPO, expressed as a percentage of the maximum bound. In the absence of unlabeled TPO, binding was 13.2% for IgG1-SP (diluted 1:1000) and 12.3% for IgG4-SP (diluted 1:30). The data are representative of two similar experiments. B and C, Scatchard analysis (27) of TPO binding by IgG1-SP and IgG4-SP.
Discussion

We have converted the TPO autoantibody Fab SP1.4, cloned from thyroid infiltrating B cells of a Graves' patient (reviewed in Ref. 6), into two complete IgG molecules of different subclass, IgG1-SP and IgG4-SP, secreted by mammalian cells. Some antibodies with the same antigen-binding regions but different constant regions have been reported to differ in their dissociation constants ($K_d$) for antigen (31). It is interesting, therefore, that IgG1-SP and IgG4-SP retain the same affinity ($K_d$) for TPO as does Fab SP1.4 ($\sim 2 \times 10^{-10}$ mol/L) (22).

Binding of IgG to FcγR requires glycosylation (reviewed in Ref. 17). Consequently, PBMC binding of IgG1-SP/[^125I]TPO complexes confirms that the mammalian SP2/0 cells secrete glycosylated IgG molecules. Unlike PBMC, no binding of complexes was observed to EBVL. Like other B cells, the EBVL only express FcγRII. PBMC, on the other hand, include monocytes, B cells, and natural killer cells that express FcγRI, FcγRII, and FcγRIII, respectively (reviewed in Ref. 17 and 32). Consequently, PBMC binding of IgG1-SP/ TPO complexes must occur either via the monocyte FcγRI and/or the natural killer cell FcγRIII.

The differential binding of IgG1-SP and IgG4-SP to PBMC...
of important features. Thus, two major types of approach have been used previously to examine IgG subclass difference in relation to ADCC. First, differential IgG1- and IgG4-mediated ADCC has been studied with polyclonal antibodies and nonnucleated erythrocytes as target cells (18). This system can be influenced by the presence in polyclonal sera of antibodies of different affinities and to different epitopes (18). The second type of investigation involves ADCC by monoclonal antibodies of different subclasses to an identical epitope, usually a small molecule (hapten), and target cells chemically coated with the hapten (35). Our study of the role of IgG subclass in ADCC involved 1) nucleated cells (such as thyocytes), which are less sensitive to lysis than are nonnucleated erythrocytes (30); 2) a large, naturally expressed protein antigen, as opposed to a small hapten chemically coupled at high density to target cells; and 3) human monoclonal autoantibodies of identical affinity and epitopic specificity. A comparison has been performed of ADCC mediated by IgG1 and IgG4 antibodies with identical V regions specific for the CDw35 antigen on T lymphocytes (36). With this exception, our study appears to be one of the few performed in which all three of these features have been present simultaneously.

The small extent of ADCC that we observed is lower than might be anticipated for a high affinity monoclonal antibody. The efficacy of an antibody in mediating ADCC cytotoxicity is influenced not only by antibody isotype, but also by epitopic density (reviewed in Ref. 17). In particular, it has been suggested that for therapeutic purposes, combinations of different murine monoclonal antibodies to different epitopes on the same antigen may be more effective than one antibody alone (reviewed in Ref. 17). Future studies will indicate if multiple monoclonal TPO autoantibodies to nonoverlapping epitopes are more effective.

The preferential effect of IgG1-SP relative to IgG4-SP in mediating ADCC is consistent with clinical observations on TPO autoantibodies in patients during the postpartum period. During this period, some TPO autoantibody-positive women develop symptoms of thyroid dysfunction suggestive of thyroid damage (for example, Ref. 37). Consequently, it is of interest that in two studies (38, 39), but not in a third (40), an association has been noted between IgG1 (but not IgG4) TPO autoantibodies and hypothyroidism.

In conclusion, a human TPO-specific Fab converted to IgG1, but not IgG4, can mediate cytotoxic effects on human thyroid cells in vitro. These observations support the clinical relevance of TPO autoantibody subclass distribution and emphasize the likelihood that, as opposed to being simple markers of thyroid damage, TPO autoantibodies may play a role in the induction of thyroid dysfunction in vivo.

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