Microparticle-Enhanced Nephelometric Immunoassay of Anti-Thyroid Peroxidase Autoantibodies in Thyroid Disorders

Asmae Alami Harchalı,1 Paul Montagne,1,3 Jean Ruf,2 Marie Louise Cuillière,1 Marie Christine Bene,1 Gilbert Faure,1 and Jean Duheille1

Crude thyroid peroxidase extracted from human thyroid microsomes was covalently bound onto polyacrylic and polyfunctional copolymerized microparticles. We observed agglutination of the thyroid peroxidase–microparticle conjugate with 13 monoclonal antibodies (mAbs) specific for epitopes on four different antigenic domains of human thyroid peroxidase (TPO; EC 1.11.1.7), after addition of anti-mouse immunoglobulins. We quantified agglutination by measuring with a specially designed nephelometer the light scattered by the conjugates. This allowed us to develop a microparticle-enhanced nephelometric immunoassay for human anti-TPO autoantibodies (aAbs) with defined epitopic specificity, based on the ability of aAbs to inhibit mAb-induced agglutination. Applied to patients with autoimmune thyroid diseases, this assay confirmed the polyclonality of anti-TPO aAbs and their preferential reactivity toward epitopes located on the A and B antigenic domains of the TPO molecule. The same specificities seem to be present in patients with Hashimoto thyroiditis or Graves disease.

Indexing Terms: autoimmune disease/monoclonal antibodies/serological diagnosis/Graves disease/Hashimoto disease

Microparticle-enhanced nephelometric immunoassays are an easy-to-perform method for measuring antigens in biological fluids (1–7). This technique has recently been applied to the detection of human anti-thyroglobulin autoantibodies (aAbs) with defined epitopic specificity in serum samples from patients with Hashimoto thyroiditis and Graves disease (8).4 We report here the application of this method to the detection of human anti-thyroid peroxidase (TPO; EC 1.11.1.7) aAbs.

Circulating aAbs directed to several components of thyroid tissue are commonly detected in the serum of patients with autoimmune thyroid diseases (9). TPO was recently identified as the major component of the microsomal antigen (10, 11) frequently recognized by such aAbs. Various RIAs and ELISAs have been developed to detect anti-TPO aAbs (12–15); the characteristics of TPO have been determined (16).

Using a panel of 13 anti-TPO monoclonal antibodies (mAbs), Ruf et al. (17) mapped four antigenic domains (A, B, C, and D) on the TPO molecule. Anti-TPO aAbs from patients with Hashimoto thyroiditis and Graves disease were found to be reacting mainly with two antigenic domains, A and B. The same panel of 13 anti-TPO mAbs was used in this work to agglutinate a microparticle–TPO conjugate specially prepared for nephelometric immunoassay. We investigated the ability of aAbs to inhibit the conjugate agglutination produced with mAbs. This allowed us to develop a microparticle-enhanced nephelometric immunoassay for detecting anti-TPO aAbs with defined epitopic specificity in serum samples from patients with autoimmune thyroid diseases.

Materials and Methods

Reagents

n-Octyl-β-D-glucopyranoside was purchased from Sigma (St. Louis, MO). Acrolein, 2-hydroxyethylmethacrylate, and methacrylic acid were obtained from Merck (Darmstadt, Germany). N,N'-Methylenebisacylamide was purchased from Eastman Kodak (Rochester, NY). Other chemicals were analytical reagent-grade from Prolabo-Rhône Poulenc (Paris, France). The buffer for nephelometry from Diagnostics Pasteur (Marnes, France) was used for the nephelometric immunoassays.

We obtained human thyroid samples in the course of therapeutic surgery from the University Hospital of Nancy (France) and stored them at −20°C before use. Crude TPO [TPO(mic)] was solubilized from the microsomal fraction (ultracentrifugation at 104 000g, 4°C, 1 h) of thyroid tissue with 40 mmol/L n-octyl-β-D-glucopyranoside in 67 mmol/L phosphate buffer, pH 7, according to Portmann et al. (11) and was stored frozen at −20°C. Protein concentration was determined in the TPO(mic) preparation according to Lowry et al. (18). Peroxidase activity was measured by the guaiacol method (19).

The 13 anti-TPO mAbs produced and characterized by Ruf et al. (17) were used as produced in ascites without further purification. These mAbs recognize seven epitopes localized in four distinct antigenic domains of the TPO molecule: A (mAbs 2, 9, 47, and 60), B (mAbs 15, 18, 59, and 64), C (mAb 24), and D (mAbs 1, 30, 40, and 58). Their protein concentration (18) ranged from 0.32 to 0.82 g/L. The Ig fraction of rabbit anti-mouse Ig antiserum, from which cross-reactivity with human Ig had been removed by solid-phase adsorption, was obtained from Dako (Glostrup, Denmark).
Samples
We obtained human sera from 120 patients with various disorders at the University Hospital of Nancy. The procedures followed were in accordance with the ethical standards of the national committee. Indirect immunofluorescence (IF) was performed with 4-μm-thick frozen-cut sections of human thyroid (20) to screen for cytoplasmic fluorescence indicative of possible anti-microsome aAbs. Anti-TPO aAb concentrations were measured in the positive samples by using an ELISA with recombinant TPO (Pin Immuno Assay; Orgadan, Mainz, Germany) calibrated against the World Health Organization reference preparation IRP 66/387. We used 30 samples to study the effect of human serum on agglutination of microsphere (MS)-TPO(mic) conjugate by mAbs. Anti-TPO aAb concentrations in these samples ranged from 6 to 6985 kIU/L. Ten samples had titers <350 kIU/L, below the threshold of positivity for the ELISA. Microparticle-enhanced nephelometric immunoassay of anti-TPO aAbs was performed in the 90 other sera. Clinical diagnoses (examination and analysis of hormone concentration) for these patients were as follows: 30 patients had Hashimoto thyroiditis (26 women and 2 men, 26–67 years old; ELISA-determined serum concentration of anti-TPO aAbs 851–7328 kIU/L, mean = 3720 kIU/L); 30 patients had Graves disease (26 women and 4 men, 23–78 years old; anti-TPO aAb concentration 383–10 360 kIU/L, mean = 2586 kIU/L); and 30 patients (26 women and 4 men, 23–85 years old; anti-TPO aAb concentration 353–2661 kIU/L, mean = 751 kIU/L) with normal results after checkup for thyroid hormones (nodular and adenomatous goiter, hypothyroidism after antithyroid treatment, thyrotoxicosis, insulin-dependent diabetes).

Procedures
Preparation of MS-TPO(mic) conjugate. Microparticles were synthesized as previously reported (8): An aqueous mixture of acrolein (64.6 g/L), 2-hydroxyethylmethacrylate (61.1 g/L), methacrylic acid (2.6 g/L), N,N′-methylenebisacrylamide (1.7 g/L), and sodium decyl sulfate (0.9 g/L) was polymerized under γ-irradiation (60Co source, 25 krad · cm−2 · h−1, 3 h). Polyacrylic MS were stored under argon at 4°C in hydroquinone (1 g/L) and were characterized (shape, size, dispersion, and concentration) as described elsewhere (21).

The MS-TPO(mic) conjugate was prepared by mixing 10 g of MS with 2 g of TPO(mic) in 1 L of 0.1 mol/L borate buffer, pH 8.2, containing NaCl, 0.3 mol/L. After an incubation of 2 h at room temperature followed by 18 h at 4°C, 50 mL of a buffered solution of 2-aminoethanol (2.4 mol/L, pH 8.2) was added to the mixture. The MS suspension was incubated for another 2 h at room temperature to block the unreacted aldehyde groups on the microparticles. The binding mixture was then ultracentrifuged (10 000g, 1 h, 4°C; Spinco L ultracentrifuge, SW50 rotor; Beckman Instruments, Brea, CA) on a discontinuous sucrose gradient (200 and 800 g/L in 0.1 mol/L borate buffer, pH 8.2, with NaCl, 0.3 mol/L). The MS-TPO(mic) conjugate was collected at the interface of sucrose solutions and was stored at 4°C in 0.1 mol/L borate buffer, pH 8.2, containing NaCl, 0.3 mol/L, and sodium azide, 2 g/L. Its concentration was calculated from dry-weight determination, and the binding yield of TPO(mic) was determined by measuring the amount of uncoupled protein in the centrifugation supernate.

Immunonephelometric reactivity of MS-TPO(mic) conjugate with mAbs. The immunoreactivity of the conjugate was tested in disposable microuettes (Nephelia cuvette; Diagnostics Pasteur) by mixing the conjugate (150 mg/L) with serial dilutions (from 1:400 to 1:102 400) of each ascitic fluid in a final volume of 0.3 mL. After 30 min at room temperature, anti-mouse Ig diluted 1:50 was added to the reaction mixture. An automated dilutor (Hamilton Bonaduz AG, Bonaduz, Switzerland) was used to perform all dilutions and dispensings in the nephelometry buffer. Conjugate agglutination was quantified by measuring with the nephelometer (Nephelia N600; Diagnostics Pasteur) previously described (1) the light scattered after 1 h of incubation at room temperature. The conjugate agglutination observed in the presence of mAb and anti-mouse Ig was inhibited by free TPO(mic) as follows: Serial concentrations of TPO(mic) from 3 mg/L to 1.7 g/L were incubated for 30 min at room temperature with mAb (ascitic fluid diluted as indicated in Fig. 1). Conjugate (150 mg/L) was added to the mixture and, after another 30-min incubation, 50-fold-diluted anti-mouse Ig was added.

Effect of human sera on conjugate agglutination by mAbs. The influence of 30 human sera on agglutination of the conjugate with mAbs and anti-mouse Ig was studied as follows: Mixtures of MS-TPO(mic) (130 mg/L) and

![Fig. 1. Inhibition by TPO(mic) of agglutination of MS-TPO(mic) conjugate (150 mg/L) in the presence of mAbs and 50-fold-diluted anti-mouse Ig (reaction time 1 h).](image-url)
serial dilutions (from 1:80 to 1:81 920) of serum samples were incubated for 1 h at room temperature in nephelometric microcuvettes. Diluted ascitic fluids (see Fig. 1) and 50-fold-diluted anti-mouse Ig were successively added after 30 min of incubation. The light scattered by the mixtures (final volume, 0.3 mL) was measured after 1 h of reaction at room temperature.

Microparticle-enhanced nephelometric immunoassay of anti-TPO aAbs in serum samples from patients with thyroid disorders. The nephelometric detection of anti-TPO aAbs in serum from patients with thyroid disorders was performed with mAbs 2, 9, 60, 15, 18, 59, and 64. Conjugate (150 mg/L) was mixed with each sample (final dilution, 1:200). After 1 h at room temperature, mAb was added (final dilution of ascitic fluid as indicated in Fig. 1) and the mixtures were incubated for another 30 min. Rabbit anti-mouse Ig (final dilution, 1:50) was finally added and the light scattered by the reaction mixtures (brought to a final volume of 0.3 mL with buffer for nephelometry) was measured after 1 h. Two controls, providing 100% inhibition (light scattered by the conjugate alone) and 0% inhibition (light scattered during agglutination with nephelometry buffer instead of serum), were included in each run. The percentage of inhibition induced by each human serum with each mAb was calculated with reference to these two controls. A 5% inhibition value was retained as corresponding to a significant decrease of the scattered light measured by the nephelometer. The light scattered at 5% inhibition was 3 SD lower than the mean of the intensity of the light scattered at 0% inhibition.

Results

MS-TPO(mic) conjugate. As previously reported (8), polyfunctional hydrophilic MS of 300 nm diameter (SD 12 nm, n = 7 measurements) were produced by copolymerization of acrylic monomers with a mean yield of 51% (SD 7%). These MS were covalently coated by TPO(mic) (peroxidase activity, 5.3 kU/g) through the formation of imine bonds between the primary amino groups of the protein and the MS aldehyde groups (21). Preliminary work, performed with various concentrations of TPO(mic) (0.25 to 8.00 g/L), had shown that the best immunoreactive conjugate was obtained when 2 g of TPO(mic) and 10 g of MS were mixed per liter of the binding mixture. In these conditions, 116 mg of TPO(mic) containing ~4 mg of pure TPO (activity of affinity-purified TPO (10), 150 kU/g) were bound on 1 g of MS.

Immunonephelometric reactivity of conjugate with mAbs. Nephelometric measurement allowed us to quantify the agglutination of MS-TPO(mic) by the 13 ascitic fluids containing different anti-TPO mAbs, in the presence of anti-mouse Ig (Fig. 2). No agglutination was observed in the absence of anti-mouse Ig (data not shown). The dilution of each ascitic fluid chosen for inhibition assay varied (see Fig. 1) according to protein concentration and agglutinating ability, and ranged between 1:400 and 1:6400. As shown in Fig. 1, conjugate agglutination with mAbs and anti-mouse Ig could be completely inhibited by 1.7 g/L TPO(mic); 50% inhibition was observed for concentrations ranging from 50 to 400 mg/L. These concentrations correspond respectively to ~60 mg/L and from 2 to 14 mg/L of affinity-purified TPO (10).

Effect of human sera on conjugate agglutination by mAbs. The effect of a human serum containing a high concentration of anti-TPO aAbs (6985 kIU/L by ELISA) on the conjugate agglutinations obtained with the 13 mAbs and anti-mouse Ig is shown in Fig. 3. Agglutination with mAbs 2, 9, and 60, which recognize the antigenic domain A on TPO, and with mAbs 15, 18, 59, and 64, which recognize antigenic domain B, was largely inhibited by high dilutions of this serum. No significant inhibition was obtained when mAbs 47, 24, and 30 (specific for antigenic domains A, C, and D, respectively) were used. The agglutination observed with mAbs 1, 40, and 53 (antigenic domain D) was only slightly altered.

Figure 4 summarizes the results obtained with each mAb for the 30 human sera used for inhibition testing at a sample dilution of 1:320. No or slight inhibition of the conjugate agglutination was observed with the 10 samples with anti-TPO aAb concentrations <350 kIU/L. For the 10 samples with aAb concentrations ranging from 350 to 2250 kIU/L, agglutination by mAbs recognizing the antigenic domains A and B of the TPO molecule were more often inhibited. The results obtained for the last 10 sera with high concentrations of aAb were similar to those shown in Fig. 3: mAbs 15, 18, 59, and 64, recognizing antigenic domain B, were strongly inhibited; the agglutination with mAbs 2, 9, and 60, recognizing antigenic domain A, was often less strongly inhibited. No or only moderate inhibition was observed with mAb 47, directed to antigenic domain A, or with
Inhibition percentage

Fig. 4. Inhibition of the conjugate agglutinations with 13 anti-TPO mAbs by human sera (320-fold diluted) containing various concentrations of anti-TPO aAbs: (top) 10 sera with aAbs concentration <350 kIU/L; (middle) 10 sera with aAbs concentration from 350 to 2250 kIU/L; (bottom) 10 sera with aAbs concentration >2250 kIU/L.

Discussion

Polyfunctional and hydrophilic microparticles produced by copolymerization of acrylic monomers have been specifically conceived as a support for antigen-antibody reactions and nephelometric markers of agglutination (21). A MS-TPO(mic) conjugate was prepared by covalent coating of such microparticles with crude

the five mAbs recognizing TPO antigenic domains C and D.

Microparticle-enhanced nephelometric immunoassay of anti-TPO aAbs in serum from patients with various disorders. Results of the nephelometric detection of anti-TPO aAbs in 90 serum samples from patients with various disorders are reported in Fig. 5. All 30 (100%) sera from Hashimoto thyroiditis patients and 17 of 30 (57%) sera from Graves disease patients strongly inhibited agglutination with at least two of the mAbs 15, 18, 59, and 64, which recognize antigenic domain B of TPO. Inhibition of this agglutination was less for another 12 (40%) samples from Graves disease patients and 19 of 30 (63%) sera from patients with normal thyroid function. In the latter group, 11 (37%) sera did not inhibit the agglutination obtained with mAbs recognizing domain B. The agglutination with mAbs 2, 9, and 60, directed to antigenic domain A of TPO, was often less significantly altered than that observed with mAbs directed to domain B: 15 (50%) and 13 (43%) sera from Hashimoto thyroiditis and Graves disease patients, respectively, induced a strong inhibition of the agglutination of at least one mAb specific for domain A. Samples from patients with normal thyroid function inhibited domain A-specific agglutination more frequently and sometimes more strongly than domain B-specific agglutination.

Polyfunctional and hydrophilic microparticles produced by copolymerization of acrylic monomers have been specifically conceived as a support for antigen-antibody reactions and nephelometric markers of agglutination (21). A MS-TPO(mic) conjugate was prepared by covalent coating of such microparticles with crude
TPO solubilized from the microsomal fraction of thyroid tissue. In spite of the poor TPO content of this TPO(mic) preparation (~3.5%, based on peroxidase activity), measurable conjugate agglutination could be obtained after a primary reaction with anti-TPO mAbs (ensuring specificity), followed by a secondary reaction with anti-mouse Ig. The anti-mouse Ig was a detector of the mAbs' binding to the conjugate, inducing agglutination and allowing nephelometric measurements. In the presence of anti-mouse Ig, 13 mAbs, recognizing four different antigenic domains on the TPO molecule (17), thus produced specific agglutination that could be completely inhibited by preincubation with free TPO(mic).

As already observed (8), no detectable agglutination of the MS-TPO(mic) conjugate could be obtained with human serum, even if it contained specific aAbs. A slight conjugate agglutination was sometimes detected at low serum dilutions, but was completely eliminated by preincubating the samples with uncoated MS. We have reported elsewhere (22) that such nonspecific agglutination could be the consequence of the interactions of defined serum proteins liable to be adsorbed on the surface of the MS.

We thus developed an assay for anti-TPO aAbs by using the ability of aAbs to impair mAb-induced agglutination. This test further provides an indication of the epitopic specificity of pathological samples. Diluted human sera containing anti-TPO aAbs, as detected by IF and quantified by ELISA, could strongly inhibit some of the conjugate agglutination obtained with anti-TPO mAbs 15, 18, 59, and 64, directed to antigenic domain B, and mAbs 2, 9, and 60, directed to antigenic domain A of the TPO molecule. This suggests a competitive reaction between the more concentrated anti-TPO aAbs and these mAbs. In comparison, the conjugate agglutination observed with mAb 47, directed to antigenic domain A of TPO, and with all mAbs that recognize antigenic domains C and D, was only slightly altered by the aAbs present in diluted human sera. These results are consistent with those already obtained by ELISA (17), showing that anti-TPO aAbs react mainly with epitopes located on TPO antigenic domains A and B. However, sera with a high concentration of anti-TPO aAbs (>2250 kIU/L) inhibited more strongly the agglutination with mAbs directed to domain B, suggesting a preponderance of aAbs directed to this domain in such samples. The infrequent inhibition by aAbs of the agglutination induced by mAb 47, the sole mAb recognizing a linear sequence of domain A (residues 713–721), also confirmed that anti-TPO aAbs are mainly directed to conformational epitopes (23).

When we applied the microparticle-enhanced nephelometric immunoassay to the detection of anti-TPO aAbs in serum from patients with autoimmune thyroid diseases, we retained a serum dilution of 1:200 in the reaction mixture as an appropriate compromise that would provide acceptable sensitivity in inhibition and avoid possible nonspecific interactions. The results of this immunoassay confirmed the polyclonality of anti-TPO aAbs already demonstrated in such disorders (14, 9).
Domain B-specific aAbs predominated in serum samples from patients with Hashimoto thyroiditis. The reverse was noted in serum samples from Graves disease patients, although the predominance of domain A specificity was not as clear. aAbs directed to domains A and B were nearly equally distributed in sera from euthyroid patients (24). The different partition of TPO domain specificity reported in this study in serum samples from Hashimoto and Graves patients does not appear as discriminant as that obtained by assessing antithyroglobulin aAbs concentrations (8). The method we describe could, however, provide complementary information in patients with autoimmune thyroiditis (24).

From the data presented here, we conclude that this application of the microparticle-enhanced nephelometric immunoassay to detect anti-TPO aAbs with defined epitopic specificity is generally suitable for serological diagnosis. The data further complement the initial report (8) of the feasibility of a comprehensive nephelometric assessment of thyroid autoimmunity.

References