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EXISTENCE OF ANTI-THYROGLOBULIN IgG IN HEALTHY SUBJECTS

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An autoantibody, anti-thyroglobulin IgG, was detected in a large proportion of healthy subjects. Sera were collected from 232 healthy subjects aged 7-83 yr, who had no apparent symptoms with normal serum levels of thyroid-stimulating hormone, confirming the absence of Graves' disease and chronic thyroiditis. Anti-thyroglobulin IgG in serum was measured by a novel enzyme immunoassay, the principle of which has been shown to provide 3,000 to 10,000-fold higher sensitivity than the conventional methods. Anti-thyroglobulin IgG was demonstrated in 38 % of the healthy subjects (15 % of those aged 7-19 yr and 69 % of those aged 20-39 yr), and the serum concentration of anti-thyroglobulin IgG was assessed to be 2 μg/l - 38 mg/l.

Autoantibodies have been demonstrated in a number of diseases with known and unknown causes, and various aspects of autoantibodies such as production mechanism, correlation to histocompatibility antigens, etiological implication and diagnostic significance have been investigated. However, autoantibodies can be demonstrated only in part of patients with autoimmune diseases, and it is little known whether autoantibodies are present in healthy subjects. This is probably due to insufficient sensitivity of currently available methods to measure autoantibodies in the circulation. We have demonstrated anti-thyroglobulin IgG in serum of healthy subjects by a novel method, the principle of which has been shown to provide 10,000-fold higher sensitivity in enzyme immunoassay for anti-insulin IgG in serum than the conventional method (1).

MATERIALS AND METHODS

Buffer

The regularly used buffer was 10 mmol/l sodium phosphate buffer, pH 7.0, containing 0.1 mol/l NaCl and 1.0 g/l bovine serum albumin (fraction V, Armour Pharmaceutical Co., Kankakee, Illinois) (buffer A).

Antibodies

Rabbit (anti-dinitrophenyl bovine serum albumin) serum was obtained from ICN ImmunoBiologicals, Lisle, Illinois. Rabbit (anti-human IgG γ-chain) IgG was obtained from Medical and Biological Laboratories Co., Ltd., Nagoya,

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Japan. Rabbit anti-human thyroglobulin serum was generously gifted from the Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan. IgG and Fab' were prepared as described previously (2), and the amount of IgG and Fab' was calculated from the absorbance at 280 nm (2).

Dinitrophenyl bovine serum albumin

1. Mercaptosuccinylated bovine serum albumin. Thiol groups were introduced into bovine serum albumin (fraction V, Armour Pharmaceutical Co.) using S-acetylmercaptosuccinic anhydride (Nakarai Chemicals, Ltd., Kyoto, Japan) (2). The amount of bovine serum albumin was calculated from the absorbance at 280 nm (3). The average number of thiol groups introduced per albumin molecule was 8.2 (2).

2. Dinitrophenyl bovine serum albumin. The mercaptosuccinylated bovine serum albumin (15 mg) in 3 ml of 0.1 mol/l sodium phosphate buffer, pH 6.0, containing 5 mmol/l EDTA was incubated with 4.5 ml of the maleimide-dinitrophenyl-L-lysine solution (1) at 30°C for 30 min. The reaction mixture was subjected to gel filtration on a column (1.5 x 45 cm) of Sephadex G-25 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) using 0.1 mol/l sodium phosphate buffer, pH 7.5. The average number of dinitrophenyl groups introduced per albumin molecule was 5.5 (4).

Protein-Sepharose 4B

Dinitrophenyl bovine serum albumin (10 mg), human IgG (10 mg), purified thyroglobulin (10 mg) and (anti-human IgG γ-chain) IgG (10 mg) were coupled to CNBr-activated Sepharose 4B (1 g) (Pharmacia Fine Chemicals AB) according to the instructions of Pharmacia.

Affinity-purification of antibodies

(Anti-dinitrophenyl bovine serum albumin) IgG and (anti-human IgG γ-chain) IgG were affinity-purified by elution at pH 2.5 from columns of dinitrophenyl bovine serum albumin-Sepharose 4B and human IgG-Sepharose 4B, respectively (5). Anti-thyroglobulin IgG (4 mg) from pooled serum of patients with Graves' disease was affinity-purified using thyroglobulin-Sepharose 4B in the same way. The amount of the affinity-purified anti-thyroglobulin IgG (5.1 μg) was measured by enzyme immunoassay (6).

Thyroglobulin and dinitrophenyl thyroglobulin

Partially purified human thyroglobulin was generously gifted from the Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan. The partially purified thyroglobulin, which had been prepared from thyroid glands by fractionation with ammonium sulfate (7), was further purified by chromatography on a column of DEAE-cellulose (8). Subsequently, the purified thyroglobulin (3.0 mg) in 1.5 ml of 0.1 mol/l sodium phosphate buffer, pH 7.0, containing 1 g/l NaN_3 was passed through a column of rabbit (anti-human IgG γ-chain) IgG-Sepharose 4B (0.9 x 5.5 cm) using the same buffer at a flow rate of 1 ml/h and subjected to gel filtration on a column (1.5 x 45 cm) of Ultrogel AcA 22 (LKB, Stockholm, Sweden) using the same buffer. Homogeneity of the purified thyroglobulin was confirmed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and urea (8). The amount of thyroglobulin was calculated from the absorbance at 280 nm (8).

Dinitrophenyl thyroglobulin was prepared from the purified thyroglobulin as described for dinitrophenyl bovine serum albumin, except that dinitrophenyl thyroglobulin was incubated with 0.5 mmol/l N-ethylmaleimide before gel filtration. The average number of dinitrophenyl groups introduced per thyroglobulin molecule was 16 (4).

Protein-coated polystyrene balls

Polystyrene balls (3.2 mm in diameter, Precision Plastic Ball Co., Chicago, Illinois) were coated by physical adsorption with affinity-purified rabbit (anti-dinitrophenyl bovine serum albumin) IgG (0.1 g/l), affinity-purified rabbit (anti-human IgG γ-chain) IgG (0.1 g/l) and the purified thyroglobulin (0.1 g/l) (9).

Fab'-peroxidase conjugates and thyroglobulin-peroxidase conjugate

Rabbit anti-thyroglobulin Fab', rabbit (anti-human IgG γ-chain) Fab' and the purified thyroglobulin were conjugated to horseradish peroxidase by reaction of maleimide groups introduced into peroxidase and thiol groups in the hinge of Fab' or thiol groups introduced into thyroglobulin (2). N-Succinimidyl-6-maleimidohexanoate was used for introduction of maleimide groups (10). The average number of peroxidase molecules conjugated per thyroglobulin molecule was 1.4. Rabbit anti-thyroglobulin Fab'-peroxidase conjugate (0.04 mg) in 0.1 ml of buffer A containing 50 mg/l thimerosal was passed through a column (3.5 x 36 mm) of human IgG-Sepharose 4B using buffer A containing 50 mg/l thimerosal at a flow rate of 0.5 ml/h. The amount of the conjugates was calculated from peroxidase activity (2).

Novel enzyme immunoassay

Test serum (20 µl) was incubated with 80 µl of buffer A containing 15 fmol of dinitrophenyl thyroglobulin, 3.75 g/l nonspecific rabbit IgG and 1 g/l NaN_3, 0.05 ml of 10 mmol/l sodium phosphate buffer, pH 7.0, containing 1.0 mol/l NaCl, 1 g/l bovine serum albumin and 1 g/l NaN_3 and two affinity-purified rabbit (anti-dinitrophenyl bovine serum albumin) IgG-coated polystyrene balls at 20°C for 4 h and at 4°C overnight. The polystyrene balls had been treated with nonspecific rabbit IgG (11). After incubation, the polystyrene balls were washed twice by addition and aspiration of 2 ml of 10 mmol/l sodium phosphate buffer, pH 7.0, containing 0.1 mol/l NaCl and incubated with 1 mmol/l dinitrophenyl-L-lysine in 0.15 ml of buffer A containing 1g/l NaN_3 at 20°C for 1 h to elute the complex of anti-thyroglobulin IgG and dinitrophenyl thyroglobulin. After removal of the polystyrene balls, the eluate was incubated with an affinity-purified rabbit (anti-human IgG γ-chain) IgG-coated polystyrene ball at 20°C for 3 h. After incubation, the rabbit (anti-human IgG γ-chain) IgG-coated polystyrene ball was washed as described above, and incubated with rabbit anti-thyroglobulin Fab'-peroxidase conjugate (50 ng) in 0.15 ml of buffer A at 20°C for 3 h. Finally, the polystyrene ball was washed as described above and bound peroxidase activity was assayed by fluorimetry at 30°C for 10 min using 3-(4-hydroxyphenyl) propionic acid as substrate (12). The fluorescence intensity was measured relative to 1 mg/l quinine in 50 mmol/l H_2SO_4 (12).

In order to more specifically test the presence of anti-thyroglobulin IgG, test serum (20 µl) was preincubated with 30 µl of buffer A containing 15 pmol of the purified thyroglobulin and 1 g/l NaN_3 at 20°C for 3 h. The reaction mixture was mixed with 50 µl of buffer A containing 15 fmol of dinitrophenyl thyroglobulin, 6.0 g/l nonspecific rabbit IgG and 1 g/l NaN_3, and 50 µl of 10 mmol/l sodium phosphate buffer, pH 7.0, containing 1.0 mol/l NaCl, 1 g/l bovine serum albumin and 1 g/l NaN_3 and was processed as described above.

Conventional enzyme immunoassays

(I). Serum containing anti-thyroglobulin IgG was diluted 1 x 10^5-fold with buffer A containing 1 g/l NaN_3, and an aliquot (0.15 ml) of the diluted serum was incubated with a thyroglobulin-coated polystyrene ball at 37°C for 3 h. After incubation, the polystyrene ball was washed as described above and incubated with rabbit (anti-human IgG γ-chain) Fab'-peroxidase conjugate (50 ng) in 0.15 ml of buffer A at 37°C for 3 h. Finally, the polystyrene ball was washed, and bound peroxidase activity was assayed as described above.

(II). Serum containing anti-thyroglobulin IgG was diluted 5 x 10^4-fold with buffer A containing 1 g/l NaN_3. An affinity-purified rabbit (anti-human IgG γ-chain) IgG-coated polystyrene ball was incubated with 0.15 ml of the diluted serum at 37°C for 3 h. After incubation, the polystyrene ball was washed as described above and incubated with 50 fmol of thyroglobulin-peroxidase conjugate in 0.15 ml of buffer A at 37°C for 3 h. Bound peroxidase activity was measured as described above.

Other methods

Measurement of anti-thyroglobulin antibodies by hemagglutination was performed using a commercial kit (Seroclit-TG, Sanko Junyaku Co, Ltd., Tokyo,

Japan). Serum levels of human thyroid-stimulating hormone (hTSH) were measured by sandwich enzyme immunoassay as described previously (13).

RESULTS AND DISCUSSION

Sera were collected from 232 healthy subjects aged 7-83 yr, who had no apparent symptoms with normal serum levels of hTSH (0.30-2.9 mU/l), confirming the absence of Graves' disease and chronic thyroiditis, and were subjected to the novel enzyme immunoassay. The fluorescence intensity for bound peroxidase activity ranged from 0.8 to 10,500 (Fig. 1). The fluorescence intensity over 1,000 was obtained by 10-fold dilution of test serum with serum which showed a fluorescence intensity of 1.4. In order to more specifically test the presence of anti-thyroglobulin IgG, an aliquot (20 µl) of all the sera, which showed fluorescence intensities over 3.0, was preincubated with 15 pmol of human thyroglobulin and subjected to the novel enzyme immunoassay. The fluorescence intensity for bound peroxidase activity was unequivocally lowered to 1.1-3.0, which were 0.12-49 % of fluorescence intensities before the preincubation, in all the sera tested. The number of sera which showed fluorescence intensities over 3.0 without the preincubation, that is, the presence of anti-thyroglobulin IgG was 89 (38 %) out of 232. The percentage of positive results was lower (15 %) in those aged 7-19 yr and higher (69 %) in those aged 20-39 yr. More positive results were observed in females than in males (Table 1). By contrast, anti-thyroglobulin IgG

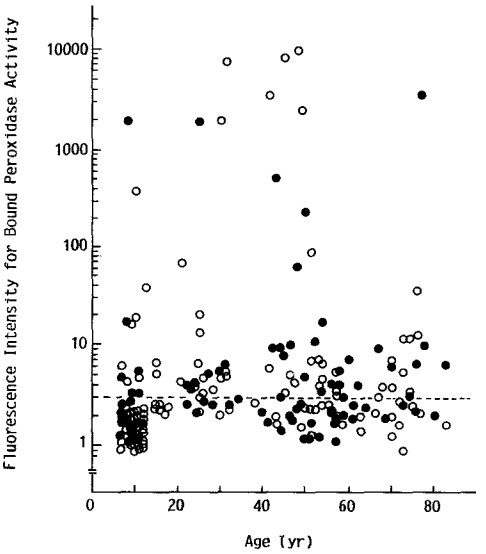

Fig. 1 Measurement of anti-thyroglobulin IgG in sera of 232 healthy subjects by a novel enzyme immunoassay technique. Closed and open circles indicate values for males and females, respectively. The broken line indicates the maximal fluorescence intensity after preincubation with human thyroglobulin.

Table 1 Number of sera which showed fluorescence intensities higher than 3.0, that is, the presence of anti-thyroglobulin IgG

Age (yr)	Sex	Number of subjects tested	Number of positive results	(%)
7-19	Male	48	6	(13)
	Female	53	9	(17)
20-39	Male	13	7	(54)
	Female	19	15	(79)
40-59	Male	33	16	(48)
	Female	29	17	(59)
60-83	Male	16	9	(56)
	Female	21	10	(48)

was demonstrated only in 0.9-1.3 % of the healthy subjects by the conventional enzyme immunoassays. The hemagglutination method gave positive results in only 1.3 %.

In order to assess the concentration of anti-thyroglobulin IgG in serum of healthy subjects, anti-thyroglobulin IgG was affinity-purified from serum of patients with Graves' disease and serially diluted with serum, which showed a fluorescence intensity of 1.4, to be subjected to the novel enzyme immunoassay. The detection limit of anti-thyroglobulin IgG in serum was 2 μg/l, and the serum concentration of anti-thyroglobulin IgG in 38 % of healthy subjects, whose sera gave fluorescence intensities over 3.0, was assessed to range from 2 μg/l to 38 mg/l.

In the conventional enzyme immunoassay methods, thyroglobulin-coated solid phase is incubated with serum and, after washing, with (anti-human IgG γ-chain) IgG-enzyme conjugate or (anti-human IgG γ-chain) IgG-coated solid phase is incubated with serum and, after washing, with antigen-enzyme conjugate. The sensitivity is seriously limited by the presence of nonspecific IgG in test serum. The use of radioisotopes as label does not improve the sensitivity. The sensitivity of the hemagglutination method is similarly low. In the novel enzyme immunoassay, nonspecific IgG is efficiently eliminated, and the sensitivity is improved 3,000 to 10,000-fold as compared with those of the conventional enzyme immunoassay methods and the hemagglutination method. As a result, anti-thyroglobulin IgG has been demonstrated in a large proportion of healthy subjects, and may be detected in all or most of healthy subjects by further improvement in the sensitivity.

It will be described in detail elsewhere that anti-thyroglobulin IgG has been demonstrated in all patients with Graves' disease (0.07-1,100 mg/l, n=16) and chronic thyroiditis (1.1-1,100 mg/l, n=8). Since the principle of the novel method appears to be applicable to the measurement of autoantibodies for most kinds of antigens, many kinds of autoantibodies will be demonstrated in significant proportions of healthy subjects. The physiological and pathological implications of autoantibodies in healthy subjects may deserve investigation from various view points.

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