HIGHLY SENSITIVE ENZYME IMMUNOASSAY OF RABBIT (ANTI-HUMAN IgG) IgG USING HUMAN IgG–β-D-GALACTOSIDASE CONJUGATE

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1. Introduction

We conjugated rabbit Fab′ to β-D-galactosidase (EC 3.2.1.23) from *Escherichia coli* by utilizing the reaction between thiol and maleimide groups [1] and showed that the sandwich enzyme immunoassay of macromolecular antigens was possible at attomole levels with the conjugates [1–3]. For example, 1 ng hepatitis B surface antigen [2] and 3.5 ng human thyroglobulin [3]/ml serum could be detected. To validate the assay of antigens at such low levels, a method is required to detect antibodies in sera at similarly low levels, since the presence of antibodies interfere with the assay of antigens.

The enzyme immunoassay of antibodies which was first reported in [4] has found a wide application to screening infectious diseases [5]. This procedure uses antigen-coated solid phase to trap antibodies and enzyme-labeled anti-immunoglobulin to measure antibodies trapped. However, the sensitivity for the assay of rabbit (anti-human IgG) IgG by this procedure was 50 ng/ml serum in our hands, being limited by the non-specific binding of normal IgG to solid materials to raise the background of assay and by some serum proteins to inhibit the binding of enzyme-labeled anti-immunoglobulin to antibodies on solid materials (serum interference).

This paper describes a highly sensitive and reproducible enzyme immunoassay of rabbit (anti-human IgG) IgG with human IgG–β-D-galactosidase conjugate by successive precipitations with anti-β-D-galactosidase and anti-rat Fc antibodies, giving a sensitivity of 0.45 ng/ml serum.

2. Materials and methods

2.1. *Materials*

Bovine serum albumin (fraction V) was obtained from Armour Pharmaceut., Chicago, β-D-Galactosidase from *Escherichia coli* was obtained as a suspension in 2.2 M $(NH_4)_2SO_4$ (5 mg/ml) from Boehringer Mannheim. CNBr-activated Sepharose 4B and Sephadex G-25 and G-150 were obtained from Pharmacia Fine Chemicals AB, Uppsala. Antisera used were obtained from Miles Labs., Kankakee, unless otherwise described.

2.2. *Buffer A*

The most frequently used buffer was 0.01 M sodium phosphate buffer, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% bovine serum albumin and 0.1% sodium azide and abbreviated to buffer A in the text. The buffer was pH 7.0, unless otherwise specified.

2.3. *Determination of the content of specific (anti-human IgG) IgG*

IgG from rabbit anti-human IgG serum was passed through a human IgG-Sepharose 4B column and the content of specific anti-human IgG was calculated from the A_{280} of the sample and effluent [6].

2.4. *Assay of β-D-galactosidase activity*

β-D-Galactosidase activity was assayed at 30°C using 4-methylumbelliferyl-β-D-galactosidase as a substrate [1], and 1 unit of the enzyme activity was defined as that which hydrolysed 1 μmol substrate/min.

2.5. *Preparation of human IgG–β-D-galactosidase conjugate*

Human IgG was reduced in the presence of EDTA [7] and conjugated to β-D-galactosidase using *N,N'-o*-phenylenedimaleimide [8]. The amount of the conjugate was expressed as units of β-D-galactosidase activity.

2.6. *Preparation of inactive β-D-galactosidase*

β-D-Galactosidase suspension (0.4 ml) was centrifuged at 1700 × *g* for 10 min, and the pellet was dissolved in 0.9 ml 0.1 M sodium phosphate buffer (pH 7.5) containing 0.15 M NaCl and 1 mM EDTA. To the enzyme solution was added 0.3 ml 2 mM *p*-chloromercuribenzoate in 0.05 M sodium phosphate (pH 6.0). The mixture was incubated at 30°C overnight and subjected to gel filtration with Sephadex G-25 using albumin-free buffer A. The enzyme activity of β-D-galactosidase thus treated was 25 μunits/50 μg and continued to decrease to 8.7 μunits/50 μg within 1 week of storage at room temperature.

2.7. *Preparation of anti-β-galactosidase sera*

The pellet from 0.5 ml β-D-galactosidase suspension was dissolved in 0.5 ml saline, subjected to gel filtration with Sephadex G-150 using saline and then used for immunization. Anti-β-D-galactosidase sera were raised in rabbits by giving 2 biweekly injections of 0.5 mg β-D-galactosidase in 0.5 ml saline emulsified with 0.5 ml complete Freund's adjuvant. The rabbits were bled 2 weeks after the last injection.

2.8. *Preparation of goat anti-rabbit Fc*

IgG (82 mg) from goat anti-rabbit IgG serum was dialyzed against 0.01 M sodium phosphate (pH 7.0) containing 0.15 M NaCl and 0.1% sodium azide and passed through a normal rabbit $F(ab')_2$–Sepharose 4B column, then through a human IgG–Sepharose 4B column using the same buffer at 2 ml/h flowrate. The column size was 0.5 × 5 cm. IgG in the effluent was used as goat anti-rabbit Fc.

2.9. *Enzyme immunoassay of rabbit anti-human IgG*

Normal rabbit serum (1 ml) was mixed with various amounts of rabbit (anti-human IgG) IgG in 10 μl buffer A and then with human IgG–β-D-galactosidase conjugate (~30 fmol, 2000 μunits) in 10 μl buffer A, and the mixture was incubated overnight. (The amount of human IgG–β-D-galactosidase used/assay was adjusted so that the ratio of β-D-galactosidase activity precipitated in the presence of anti-human IgG to that in its absence could be maximal.) After the incubation, 50 μg inactivated β-D-galactosidase in 165 μl buffer A and 500 μg rabbit anti-β-D-galactosidase $F(ab')_2$ in 55 μl buffer A were added successively. The mixture was incubated at 37°C for 2 h and allowed to stand overnight at 4°C. The precipitate formed was collected by centrifugation and washed 3 times with 1 ml of chilled azide-free buffer A, then suspended in 100 μl of azide-free buffer A (pH 7.5).

To the suspension was added 12 μl azide-free buffer A containing 100 mM 2-mercaptoethylamine and 5 mM EDTA to solubilize the precipitate. After incubation for 90 min at 37°C, 4 μl azide-free buffer A containing 100 mM *N*-ethylmaleimide was added 3 times at a 20 min interval at 30°C and the mixture incubated with 2 μl 10% NaN_3 for 20 min at 30°C to facilitate the decomposition of excess maleimide. After solubilization, 6 μg normal rabbit IgG in 20 μl buffer A and 150 μg goat (anti-rabbit Fc) $F(ab')_2$ in 20 μl buffer A was successively added to the solution and the mixture was incubated for 1 h at 37°C and allowed to stand overnight at 4°C. The precipitate was washed as above, suspended in 100 μl buffer A, transfered to a β-D-galactosidase-free tube and subjected to the assay of β-D-galactosidase activity. Otherwise, the precipitate was washed and solubilized as above except that 20 μl azide-free buffer A containing 100 mM 2-mercaptoethylamine and 5 mM EDTA was added. The solubilized material was precipitated again by the addition of 6 μg normal rabbit IgG in 20 μl buffer A and 1 mg goat (anti-rabbit Fc) IgG in 70 μl buffer A. The precipitate was washed, suspended in 100 μl buffer A, transfered to a β-D-galactosidase-free tube and subjected to the assay of β-D-galactosidase activity.

2.10. *Other methods*

IgG was prepared by fractionation with Na_2SO_4 [9] and chromatography with DEAE cellulose [10]. $F(ab')_2$ was prepared by digestion of IgG by pepsin [11]. The amount of IgG, $F(ab')_2$ [6] and β-D-galactosidase [8] was calculated from A_{280}. IgG or $F(ab')_2$ (10 mg) was coupled to CNBr-activated Sepharose 4B (1 g) following the instruction of Pharmacia.

Unless otherwise specified, experiments were performed at room temperature (24–26°C).

3. Results and discussion

As shown in fig.1, a highly sensitive and reproducible assay of rabbit (anti-human IgG) IgG was possible, and the measurable range was 0.45–15 ng specific (anti-human IgG) IgG/ml serum. The single precipitation with anti-rabbit Fc enabled to determine 1.5 ng specific (anti-human IgG) IgG/ml serum. The double precipitations increased the ratio of β-D-galactosidase activity precipitated in the presence of excess antibodies to that in their absence from 4.3–10.3 and enhanced the sensitivity to 0.45 ng/ml serum. This sensitivity was ~100-fold higher than that obtained by the method [4] as described in section 1.

The precipitation with a mixture of antibodies for different immunoglobulin classes may enable us to determine the total content of anti-human IgG in serum. The content of anti-human IgG of each immunoglobulin class may be determined by the precipitation with each corresponding antibody, where the amount of human IgG–β-D-galactosidase conjugate required/assay depends upon the content of anti-human IgG in serum. An analogous procedure with antibody Fab′–β-D-galactosidase conjugate may offer a sensitive assay of antigens and immune complexes.

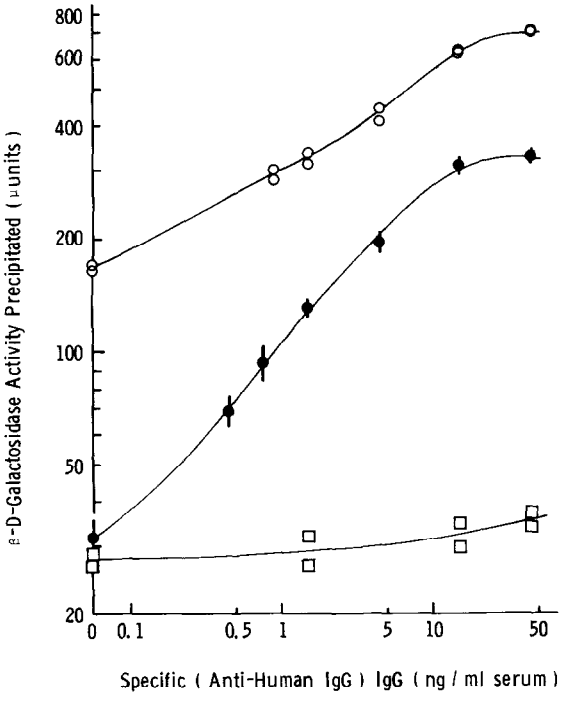

Fig.1. Enzyme immunoassay of rabbit (anti-human IgG) IgG using human IgG–β-D-galactosidase conjugate and goat anti-rabbit Fc. Open and closed circles indicate assays by the single and double precipitations, respectively, with anti-rabbit Fc, and vertical bars indicate SD from 5 expt. Open squares indicate the assay in which anti-human IgG in serum was preincubated with excess human IgG (300 ng) at 30°C for 6 h.

This procedure is somewhat tedious, requiring repeated precipitations and washings. In addition, β-D-galactosidase activity was not fully recovered in the precipitates subjected to the assay of the enzyme activity. The recovery of the enzyme activity by precipitation with anti-β-D-galactosidase was 74.0 ± 2.8 (SD, n = 42)%. The recoveries of the enzyme activity by the first and second solubilizations were 54.5 ± 2.0 (SD, n = 17) and 55.0 ± 3.6 (SD, n = 15)%, respectively. The recoveries by the first and second precipitations with anti-rabbit Fc in the presence of excess anti-human IgG (50 ng) were 93.7 ± 3.2 (SD, n = 8) and 79.5 ± 2.7 (SD, n = 4)%, respectively. Therefore, this procedure, although highly sensitive and reproducible, remains to be simplified using an enzyme resistant to the treatment with 2-mercaptoethylamine and *N*-ethylmaleimide.

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