Development of Enzyme-Linked Immunospecific Assay (ELISA) for the Detection of Monoclonal Antibody: Application to Detection of Monoclonal Anti-TBG

YUICHI MORI*, **, HISAO SEO*, TOSHIHIKO WAKABAYASHI*,
MASAAKI NAGATA***, YOSHIHARU MURATA*, NOBUO MATSUI*,
KAZUYUKI YAMAUCHI**, AKIO TOMITA**

*Department of Metabolism and Endocrinology, The Research Institute of
Environmental Medicine, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464

**The First Department of Internal Medicine, Nagoya University School of
Medicine, 65, Tsurumai-cho, Showa-ku, Nagoya 466 ***Koseiren
Kamo Hospital, 3-30-1, Nishiyama-cho, Toyota 471

Abstract

An enzyme-linked immunospecific assay (ELISA) was developed to screen monoclonal antibody to human thyroxine-binding globulin (TBG). The assay is based on the absorption of TBG from human pooled serum by rabbit polyclonal anti-TBG antibody coated on a microtiter plate and subsequent binding of monoclonal antibody to the absorbed antigen. Monoclonal antibody which binds TBG can be detected by peroxidase labeled anti-mouse IgG.

From the examination with two different rabbit polyclonal anti-TBG antibodies, it was demonstrated that both purified IgG fraction and whole serum could be used as coating materials. It was not necessary to use purified TBG to react with anti-TBG antibody coated on the microtiter plate. When eight commercially available microtiter ELISA plates were tested in the assay, only 3 gave satisfactory results. The sensitivity of the assay was comparable with that of the conventional immunoprecipitation method using ^{125}I -TBG and formalin fixed *Staphylococcus aureus* (Kowan strain) as an immunoabsorbent. The ELISA method could detect antibody activity in $0.032\ \mu\text{l}$ of medium obtained from a 3 day culture of confluent hybridoma cells. It is possible to store the antibody-antigen complexed microtiter plate for more than 2 weeks at 4 C. This makes possible rapid screening of monoclonal antibody.

Monoclonal antibodies to peptide hormones or receptors have been powerful tools in studying the relationship between structure and biological function (Andre *et al.*, 1980; Green *et al.*, 1980; Kunder *et*

al., 1983; Moyle *et al.*, 1982; Wright *et al.*, 1982).

While we raised monoclonal antibody to TBG to study its structure and function, a sensitive and rapid screening method for monoclonal antibodies was developed.

The method employs the fixation of

polyclonal antibody on the microtiter well, which then absorbs the antigen from biological fluid such as serum. Monoclonal antibody then binds to the absorbed antigen and is detected by enzyme-linked anti-immunoglobulin.

In this report the optimal conditions and sensitivity of this assay were studied.

Materials and Methods

1. Reagents.

Bovine serum albumin (BSA) was purchased from Rhes Chemical Co., USA. Lactoperoxidase was purchased from Boehringer Mannheim Co., West Germany. ^{125}I was obtained from Amersham, England. Formalin fixed staphylococcus aureus of Kowan strain (Pansorbin) was obtained from Calbiochem-Behring, USA. Orthophenylenediamine was purchased from Tokyo Kasei Kogyo Co., Ltd., Japan. Polyoxyethylene (20) sorbitan monolaurate (PE20) was obtained from Wako Pure Chemical Industries, Ltd., Japan. Rabbit anti-mouse immunoglobulin G antibody (heavy and light chain specific) was obtained from Cappel Laboratories Inc., Ca., USA. Protein A Sepharose, DEAE Sephadex A50 and Sephadex G100 were obtained from Pharmacia Co., Sweden. All other reagents were of analytical grade.

2. Preparation of monoclonal antibody to TBG.

A purified TBG preparation for immunization was a gift from Dr. J. S. Marshall (Case Western Reserve University, Cleveland, Ohio, USA.).

BALB/C mice were immunized by subcutaneous injections of the TBG preparation using Klebsiella 03 lipopolysaccharide as an adjuvant. The latter was a gift from Dr. N. Kato (Nagoya University, Nagoya, Japan) and has been shown to have a potent adjuvant activity (Ohta *et al.*, 1982).

After the 4th immunization, antibody activity was detectable in the sera of the mice at a dilution of 10,000 times by an immunoprecipitation method which will be described below. The last (5th) immunization was done intraperitoneally and 3 days after, the spleen cells were obtained from the immunized mice and were hybridized with mouse myeloma cells (NS-1 strain) (Köler and Milstein 1975). Hybridomas were selected by

HAT (hypoxanthine / aminopterin / thymidine) medium and cloned by limiting dilution (Köler and Milstein 1975).

The culture medium was changed every 3 days. When hybridoma cells became confluent, the media of the last 3-day culture were saved and used for the monoclonal antibody screening.

At the time of the spleen removal, blood samples were collected by cardiac puncture and sera were kept at -20°C until use. These mouse polyclonal anti-TBG sera were used to evaluate the method.

3. Immunoprecipitation method to detect monoclonal antibody.

TBG was labeled with ^{125}I using the lactoperoxidase method (Rogol and Rosen 1974).

In the assay, twenty microliters of hybridoma culture medium was incubated with $100\ \mu\text{l}$ ^{125}I -TBG (20,000 cpm) in phosphate buffered saline pH 7.4 (PBS) containing 1% BSA. Following overnight incubation at 4°C , $10\ \mu\text{l}$ of 10% pansorbin was added and incubated at room temperature for 30 min (Kessler 1976). After the addition of 1 ml PBS, the reaction mixture was centrifuged and the radioactivity of the precipitate was counted.

4. Purification of immunoglobulin G (IgG) fraction from rabbit polyclonal anti-TBG antiserum.

IgG fraction of a rabbit polyclonal anti-TBG antiserum was purified by affinity chromatography using protein A Sepharose (Ey *et al.*, 1978). The antiserum was applied to protein A Sepharose column and washed with 20 mM sodium phosphate buffer pH 7.4, IgG fraction was eluted with 0.1 M glycine buffer, pH 3.0. The fraction was immediately neutralized with 1 M Tris HCl, pH 8.6 and dialyzed against PBS. Finally the IgG fraction was stored at -20°C in 50% glycerol. The antibody activity in the IgG fraction was adjusted to that of the original anti-TBG serum.

5. Preparation of crude TBG from pooled human serum.

For the evaluation of ELISA, crude TBG was prepared by ammonium sulfate precipitation (30–55%), and subsequent chromatography on DEAE Sephadex A50 and Sephadex G100 (Pensky and Marshall 1969). The preparation was enriched with TBG approximately 280 times from pooled serum.

6. ELISA method to detect monoclonal antibody.

The standardized method will be summarized below.

i) Coating of the microtiter well with rabbit polyclonal anti-TBG.

One hundred microliters of rabbit polyclonal anti-TBG, diluted 1:1,000 in 0.1M sodium carbonate buffer pH 9.1, was incubated overnight at 4°C in the well of Costar 3590 microtiter plates. The wells were washed six times with PBS containing 0.05% PE20 (Solution A).

ii) Binding of TBG to the fixed polyclonal anti-TBG.

One hundred microliters of pooled serum diluted 1:50 in PBS containing 1% BSA and 0.05% PE20 was added to the well. After 1 hour incubation at room temperature, the plates were washed six times with Solution A.

iii) Binding of mouse monoclonal anti-TBG to the absorbed TBG.

Thirty microliters of hybridoma culture medium was added to the microtiter well and incubated at room temperature for 1 hour. The plates were washed six times with Solution A. To evaluate the method, 100 μ l of polyclonal mouse anti-TBG at various dilutions was used instead of culture medium.

iv) Detection of monoclonal antibody with peroxidase labeled anti-mouse IgG.

One hundred microliters of peroxidase labeled

rabbit anti-mouse IgG, diluted 1:500, in PBS containing 1% BSA and 0.05% PE20 was added to the well and incubated at room temperature for 1 hour. After washing six times with Solution A, color development was initiated by adding orthophenylenediamine 0.02% and hydrogen peroxide 0.005% in 0.1M citrate buffer pH 4.0 (100 μ l). After incubation for 1 hour at room temperature, the reaction was stopped by adding 50 μ l 4M sulfuric acid. Optical density of the reaction mixture was measured at 450 nm using an Immunoreader from Nippon Intermed Co., Ltd., Japan.

Results

1. Coating of the microtiter well with rabbit polyclonal anti-TBG.

Two different rabbit polyclonal anti-TBG antisera were tested as coating materials. IgG fraction purified from one of the antisera was also used. When 100 μ l of antiserum or purified IgG fraction with at different dilutions were used to coat the microtiter well, purified IgG fraction gave the highest OD reading at a dilution 1:200 to 1:2000 (Fig. 1). It should be noted that

Fig. 1. Influence of the amount of rabbit polyclonal anti-TBG antibody on the final reaction.

Two different rabbit polyclonal anti-TBG sera (No. 1 and No. 2) as well as IgG fraction of No. 1 antiserum were diluted in 0.1M sodium carbonate buffer pH 9.1. The source of TBG was 100 μ l of pooled human serum diluted 1:50. Mouse anti-TBG 100 μ l was polyclonal and diluted 1:1000. One hundred microliters of peroxidase labeled anti-mouse IgG diluted 1:500 was used as described in Materials and Methods.

subsequent steps were performed as described in methods. When whole anti-sera were used, the OD reading was almost constant from a dilution of 1:200 to 1:4000, and it was slightly lower than the OD reading obtained with the purified IgG fraction.

Incubation time for the coating with the antibody was tested at 4 C for various length of time. Incubation from 16 hours to 72 hours gave identical results (data not shown). Thus, in the standard assay, the microtiter well was coated with 100 μ l of unpurified anti-TBG, diluted 1:1000 overnight at 4 C.

2. TBG binding to rabbit polyclonal anti-TBG coated on the microtiter well.

To evaluate the binding of TBG to the polyclonal anti-TBG coated on the microtiter well, a constant amount of 125 I-TBG (20,000 cpm) and 100 μ l of pooled serum at various dilutions were added to the well of polyvinylchloride (PVC) microtiter plate (Cooke Co., USA). Precoating was achieved with rabbit polyclonal anti-TBG at a dilution

of 1:1000. After incubation for 1 hour, the plates were washed and the radioactivity of each well was counted. As shown in Fig. 2, the binding sites on the well were almost completely saturated with the pooled serum at a dilution of 1:50. Since there was no radioactivity of 125 I-TBG absorbed on the well without precoating the microtiter well with rabbit polyclonal anti-TBG antibody, nonspecific binding of TBG on the microtiter well was negligible. The amount of TBG absorbed on the microtiter well was not different when the well was coated with rabbit polyclonal anti-TBG diluted from 1:200 to 1:4000 and incubated with the pooled serum at a dilution of from 1:5 to 1:200. Since the pooled serum contained 16 μ g/ml TBG, the TBG binding capacity was 32 ng/well. Crude TBG instead of diluted serum gave almost the same results as the same input of TBG. Moreover, incubation of complete TBG deficient serum with rabbit polyclonal anti-TBG coated on the microtiter well did not give an appreciable OD reading, so that nonspecific

Fig. 2. Influence of the amount of TBG on the absorption of TBG to rabbit polyclonal anti-TBG.

After coating the microtiter wells with rabbit polyclonal anti-TBG, 125 I-TBG (20,000 cpm) and 100 μ l of human pooled serum with various dilution were added to the wells. The radioactivity bound to the well was counted.

The arrow in the figure indicates the dilution of human serum used in the standard assay.

absorption of serum proteins other than TBG did not seem to interfere with this assay system. Consequently, pooled serum was used as a source of TBG.

There was no decrease in the OD reading after the storage of the polyclonal antibody-antigen complexed microtiter plate for 2 weeks at 4 C.

3. Concentration and incubation time of peroxidase labeled rabbit anti-mouse IgG.

After coating the microtiter well with rabbit polyclonal anti-TBG and reaction with TBG and subsequent binding of mouse anti-TBG, peroxidase labeled rabbit anti-mouse IgG at a different dilution was reacted for 1 hour. After washing, the substrate was added and OD at 450 nm was measured. Almost maximal OD at 450 nm was obtained with 100 μ l peroxidase labeled antibody, diluted 1:500.

One hour incubation with peroxidase labeled anti-IgG was sufficient for the

binding of mouse IgG. Thus, the reaction of peroxidase labeled anti-IgG was carried out for 1 hour at room temperature.

4. Substrate concentration and incubation time for peroxidase reaction.

When the optimum substrate concentration and pH were tested, color development was highest at pH 4, at a concentration of orthophenylenediamine 0.02% and at a concentration of H_2O_2 0.005%. One hour of incubation with the substrate was also found satisfactory.

When absorbance figures were compared at different wavelengths (from 414 to 620 nm), the highest reading was obtained at 450 nm.

5. Selection of ELISA plate.

Eight different commercially available plates were tested. As shown in Fig. 3, the plates from Costar (Serocluster EIA plate flat bottom 3590) and Nunc (Immuno

Fig. 3. Comparison of commercially available microtiter plates for ELISA.

Eight different ELISA plates were tested in the standard assay using different amounts of mouse polyclonal anti-TBG.

The plates examined were as follows, 1: Costar ELISA (3590) 2: Nunc ELISA 3: Costar PVC 4: Cooke PVC (U shape) 5: Cooke PVC (flat shape) 6: Falcon ELISA (3042) 7: Sumitomo ELISA (H) 8: Sumitomo ELISA (F3496)

Fig. 4. Comparison of sensitivity of ELISA and immunoprecipitation method using mouse polyclonal anti-TBG.

Sera from mice immunized with TBG were diluted serially. Antibody titer was determined by ELISA (○---○) and immunoprecipitation method (●—●).

Fig. 5. Comparison of sensitivity of ELISA and immunoprecipitation method using mouse monoclonal anti-TBG.

Culture medium of a hybridoma clone producing anti-TBG was serially diluted and antibody titer was determined by both ELISA and immunoprecipitation method.

Plate II) were the best and PVC plates (Cooke U shape, Cooke flat shape and Coster U shape) gave satisfactory results.

The other ELISA plates gave a very low OD reading.

6. Comparison of ELISA and immunoprecipitation method.

When mouse polyclonal anti-TBG at different dilutions was tested in both ELISA and immunoprecipitation assay, it was found

that both assays could detect antibody activity in a similar fashion (Fig. 4).

7. Screening of monoclonal antibody.

Culture media of 326 hybridoma clones were screened by both the ELISA and immunoprecipitation method. Two positive clones were identified by both methods. When one of the monoclonal antibodies produced in culture medium was diluted

serially and assayed with both methods, the detection sensitivity was almost identical (Fig. 5). Actually antibody activity was detected in less than 0.032 μ l of culture medium.

Discussion

One of the most important prerequisites for the production of monoclonal antibody is to have a simple, rapid and sensitive screening method.

The use of antigen labeled with radioisotope certainly fulfils this requirement. However, screening of more than 500 hybridoma clones with conventional immunoprecipitation method requires more than 24 to 48 hours because of the centrifugation and counting. Also the introduction of 125 I to the antigen could inhibit the binding of monoclonal antibody.

The ELISA assay reported here has sensitivity similar to the immunoprecipitation method. Moreover, actual screening of more than 500 hybridoma clones can be completed in 4 hours when the antibody-antigen complexed microtiter plate is prepared before the assay.

Theoretically, anti-TBG antibody activity could be assayed in a microtiter well coated with the antigen (TBG) itself. However, conventional techniques used in purification of a large amount of TBG are difficult and cumbersome. The introduction of polyclonal antibody makes it unnecessary to purify a large amount of antigen.

Recently published results (Brock *et al.*, 1984) indicated that the quality of the polyclonal antibody used to coat the microtiter well was an important factor in binding the antigen. In our assay, at least two different rabbit polyclonal anti-TBG preparations were satisfactory.

It was not expected that some of the commercially available microtiter plates specifically sold for ELISA could not be

used in our assay. It seems possible that binding of rabbit polyclonal antibody to the microtiter well varies from one brand to the other. Thus, the selection of suitable microtiter plates is very important.

As mentioned in the report by Brock *et al.* (1984), the use of rabbit peroxidase labeled anti-mouse IgG is also important in avoiding cross reactions with polyclonal antibody coated on the well.

Although it is possible that monoclonal antibody which shares the major antigenic site with polyclonal antibody coated on the well could not be detected in the present assay, actual screening of 326 hybridoma culture media resulted in 2 positive clones by both ELISA and the immunoprecipitation method.

The assay should be generally applicable if suitable polyclonal antibody is available.

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