A COMPETITIVE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETERMINATION OF HUMAN ALBUMIN IN THE NANOGRAM RANGE

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SUMMARY :

Purpose: *Human serum albumin (HSA)-specific IgG1 monoclonal antibody (mAb) producing hybridoma clone (A-HAlb/98) was produced in our laboratory. The aim of this study was to make a competitive enzyme-linked immunosorbent assay to measure nanogram levels of human albumin in samples with the use of anti-HSA IgG1 mAb.* ***Method:*** *High-binding capacity flat-bottomed 96-well microtitre plates were coated with HSA or bovine serum albumin (BSA) and then blocked with gelatin. Standard HSA solutions and diluted serum samples were added into wells in the presence of anti-HSA. After incubation, the plate was washed and enzyme-labeled anti-mouse IgG added. Following an additional incubation period, the plate was re-washed and chromogen-substrate added. Reaction was stopped and evaluated with an ELISA reader. Comparison was made with nephelometric measurement.* ***Results:*** *A-HAlb/98 hybridoma-produced IgG1 mAb specifically bound to HSA. It did not react with BSA. With the competitive ELISA, dynamic measuring range of human albumin in samples was found to be between 15.6 - 1000 nanogram per 100 µl. Nearly 250 times lower concentrations of albumin could be measured with competitive ELISA when compared to minimum albumin levels detected with Beckman nephelometry in the cerebro spinal fluid (CSF) mode. Results obtained by competitive ELISA correlated significantly with those from parallel experiments with nephelometric assay.* ***Conclusion:*** *The competitive ELISA described is a sensitive assay for determination of albumin in the nanogram range and may be a promising method for routine analysis of albumin concentrations especially at low levels or to survey microalbuminuria which is a marker strongly predictive of diabetic nephropathy.*

Key Words : *Enzyme-Linked Immunosorbent Assay, Serum Albumin, Monoclonal Antibodies.*

INTRODUCTION

A monoclonal antibody (mAb) is directed against a single antigenic determinant, the epitope. Therefore, it possesses a unique specificity and an extremely high selectivity for the epitope. The development of hybridoma technology (1), for the production of specific and high-affinity monoclonal antibodies has provided invaluable probes for antigenic analyses. Monoclonal antibodies have made a triumphal entry into immunological and immunohistological diagnostics (2-5). Monoclonal antibodies have improved both the diagnosis of viral and bacterial diseases and the mAb-based ELISA kits which are now commercially available for enzymes, tumor markers, hormones, and cell surface antigens

increase in number almost daily (6-8).

We recently reported (9) the establishment of hybridomas (CAy-M and CAy-G) secreting monoclonal IgM and IgG1 antibodies which specifically recognized hepatitis B virus surface antigen (HBsAg). In that study immunization was performed with the serum-isolated HBsAg. Isolation of HBsAg frequently results in contamination with albumin (10,11). Thus, some of the hybridomas established in our laboratory were found to secrete mAb specific for human serum albumin (HSA). One of them, called A-HAlb/98, was cloned at single-cell level and produced in large quantities and then frozen in liquid nitrogen.

We thawed one vial of A-HAlb/98, cultured in a medium to let them secrete anti-HSA mAb, and described herein their utilization for a competitive ELISA for measuring low levels of albumin in samples.

MATERIALS AND METHODS

Human serum albumin specific mouse IgG1 mAb-producing hybridoma clone (A-HAlb/98) was generated by one of our authors. It was a by-product obtained during HBsAg-specific mAb production study (9). Because the immunization was made with the serum-isolated HBsAg, contaminant HSA caused generation of HSA-specific B cell blasts and the anti-HSA mAb producing hybridomas at the fusion step. A-HAlb/98 hybridoma cells (1x106 cells/ml) were cultured for 72hr in 250ml tissue culture flasks containing RPMI-1640 (Sigma) supplemented with 10 % heat-inactivated fetal calf serum (FCS, Biochrom). Supernatant was centrifuged and passed through a 0.45 µm pore-sized filter (Semadeni) and then diluted either with RPMI-1640 or PBS-Saline (10mM phosphate buffer with 154mM NaCl, pH 7.3) at the desired titration.

Human serum albumin (fatty acid and globulin free, Sigma), bovine serum albumin (BSA, Sigma) and gelatin (Merck) were dissolved in carbonate-bicarbonate buffer (CBB, 15mM Na_2CO_3 plus 35mM $NaHCO_3$, pH 9.6) at the concentrations of 10 µg/ml, 10 µg/ml, and 0.3 % respectively. Coating of the flat-bottomed 96-well microtitre plate (high binding capacity ELISA plate, Greiner) was made by adding 50 µl of the prepared reagents per well and incubating for 2 hr at 37°C. After washing the plates with washing buffer (PBS-Saline containing 0.05% Tween 20), wells were blocked with 300 µl of blocking buffer (CBB containing 0.05% Tween 20, 1mM EDTA, 0.3 % gelatin and 0.05% NaN_3) by keeping overnight at +4°C (12).

In order to demonstrate specific binding of A-HAlb/98 mAb, 100 µl of titrated supernatant was added into HSA-, BSA-, or gelatin-coated wells. The plate was incubated for 1hr at 37°C, washed 6 times, 100 µl of 1/3000 diluted HRPO-labeled anti-mouse IgG conjugate (Sigma) added and then incubated for 1 hr at 37°C, again washed 6 times, 200 µl of TMB chromogen-substrate (tetramethylbenzidine, 0.009 % H_2O_2 and 4 % dimethyl sulfoxide) added and incubated for 20 min at room temperature (RT) and the reaction was then stopped by adding 100 µl of 1.5N H_2SO_4 solution. Optical densities (OD, or absorbance) was measured with an ELISA reader (LP400 Diagnostics Pasteur) at 450 nm with a 620 nm reference filter.

For competitive ELISA, after washing the plates 6 times, 100 µl of standards (HSA prepared in PBS-Saline at the concentrations from 0.156 (g/ml to 10 µg/ml) or 1/10.000 diluted (in PBS-Saline) 20 serum samples were added to the wells. This was followed by addition of 100 µl of 1/12 diluted A-HAlb/98 culture supernatant. The plate was incubated for 1 hr at 37°C. Conjugate, substrate and stop solution adding steps were performed as described above.

For comparison of serum albumin levels obtained by competitive ELISA to another method, serum albumin levels were also measured with nephelometric assay (Beckman protein array system).

RESULTS

With a commercially available isotyping kit (ISO-1, ImmunoType, Sigma), it was found out that A-HAlb/98 mouse hybridoma cells produced mAb in IgG1 isotype. A-HAlb/98 hybridoma-produced mAb (anti-HSA) specifically bound to human serum albumin and did not react with BSA (Fig. 1). Binding of anti-HSA to HSA-coated wells was observed to be concentration dependent (r=0.9526, p=0.0123)

During measurement of albumin levels with competitive ELISA, it was observed that the

dynamic measurable range was between 15.6 nanogram/100µl and 1000 nanogram /100µl (Fig. 2). There is significant negative correlation (r= -0.9002, p=0.0023) in that range. The reaction was inversely correlated with the amount of free albumin in standards or samples. Free albumin in samples blocked the anti-HSA IgG1 and inhibited it from binding to coated HSA and then was washed away in the washing step. This in turn decreased the binding of enzyme-labeled anti-IgG and diminished the OD levels produced by enzyme-substrate reaction. Although high concentrations of BSA (0.5-1µg/100µl) decreased the reaction to a lesser extent, it was evaluated as a non-specific interaction because anti-HSA did not bind to BSA-coated wells (Fig. 1).

Albumin levels of 20 serum samples were measured with a nephelometric assay (Beckman, Protein Array System). The same serum samples were diluted to 1/10.000 with PBS-Saline and also studied with the competitive ELISA. The mean absorbance of 1/10.000 diluted duplicate samples, obtained from competitive ELISA, was plotted against a standard curve (Fig. 2) to assess the albumin level, multiplied by a dilution factor and then converted to mg/dl. Although serum albumin levels measured with competitive ELISA were found to be higher than that obtained with nephelometric measurment, there was a significant correlation (r=0.9393, p<0.00001) between these two assays (Fig. 3).

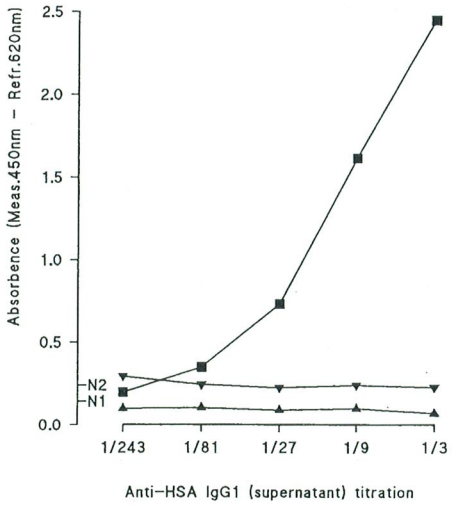

Fig. 1 : Specificity of A-HAlb/98 mAb against human albumin. 96-well flat-bottomed microtitre plate was coated either with HSA (■.50µl (10µg/ml) per well), BSA (▲, 50µl (10µg/ml) per well), or gelatin (▼, 50µl (0.3%) per well) for 2h at 37°C, and then blocked with gelatin (300µl of 0.3% solution per well). After washing the plate, A-HAlb/98 hybridoma culture supernatant was titrated with RPMI and added into wells in a volume of 100µl. After incubation for 1h at 37°C, the plate was washed and then incubated for an additional 1hr in the presence of 100µl of conjugate. After re-washing, substrate was added and then 20 min later, reaction was stopped. Plate was read with an ELISA reader. Concentration-dependent (r=0.9526, p=0.0123) binding of A-HAlb/98 hybridoma supernatant to wells was observed only with the HSA-coated wells. N1 denotes negative control (complete medium) value of HSA- and BSA-coated wells. N2 denotes negative control (compete medium) value of gelatin-coated wells. Data are the mean of duplicate wells.

DISCUSSION

The experimental results presented here described the production of human albumin-specific mAb-producing hybridoma cells (A-HAlb/98). These experiments also described anti-HSA mAb-based competitive ELISA for quantification of human albumin in serum samples.

A-HAlb/98 recognizes natural human serum albumin. This is apparent from the demonstration with direct ELISA that the mAb binds to HSA-coated wells in a concentration-dependent manner.

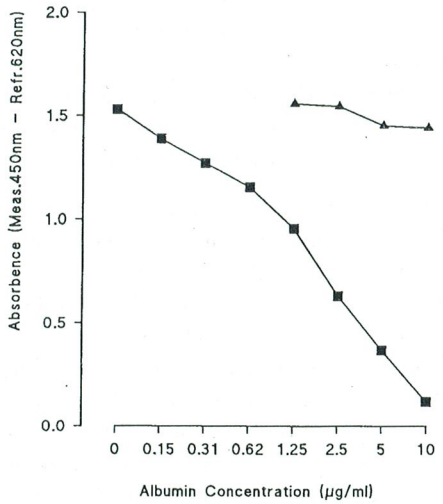

Fig. 2 : Competitive ELISA for measuring albumin concentration. 100 µl of 1/12 diluted (with PBS-saline) A-HAlb/98 hybridoma culture supernatant and 100µl of HSA (■) or BSA (▲) of varying concentrations were added to human albumin coaled wells. After incubation at 37°C for an hour, plate was washed and then incubated for an additional 1hr the presence of 100µl of conjugate. After washing, 200µl of substrate was added into each well, incubated for 20 min at RT, and then reaction was stopped by adding 100µl of stop solution per well. Absorbance values were read with an ELISA reader. Dynamic inhibitory effect (r=-0.9002, p=0.0023) of HSA was observed between 15-1000ng/100µl. Concentration-dependent inhibitory effect did not occur with BSA even at high concentrations (500 or 1000ng). Data are the mean of duplicate wells.

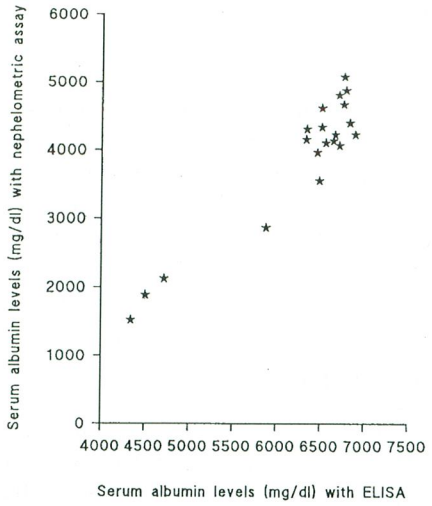

Fig. 3 : Comparison of the competitive ELISA and nephelometric assay for human serum albumin levels. 20 serum samples were measured for their albumin contents with nephelometric assay (Beckman) and competitive ELISA. For competitive ELISA, serum samples were first diluted to 1/10.000 and then OD values were plotted against standard curve (Figure 2) to find albumin contents. The results were multiplied with the dilution factor and then converted to mg/dl. Significant correlation ($r=0.9393$, $p<0.00001$) between competitive ELISA and nephelometric measurements was observed. Data of the competitive ELISA are the mean of duplicate wells.

The described mAb can selectively discriminate human albumin from bovine albumin because it did not react with BSA even at higher concentrations.

With the competitive ELISA it was observed that human albumin in the nanogram range could be easily detected. Absorbance value was significantly correlated ($r=-0.9002$, $p=0.0023$) with the amount of albumin between 15.6 and 1000 nanogram per 100 µl. With the Beckman nephelometric protein analyzer (Albumin, Array protein system), minimum measurable albumin level is 800 mg/dL (8 mg/ml) for serum samples and 3.7 mg/dL (37 µg/ml) for cerebrospinal fluid (according to the manufacturer's manual handbook and to the prospectus of the albumin kit). When compared to Beckman nephelometric measurement, even at CSF mode, nearly 250 times lower concentration of albumin could be measured with competitive ELISA described in this study. This is especially important when low levels of albumin is needed to be measured, and such conditions were reported to be needed by many investigators (13-20).

20 serum samples were assessed for their albumin levels with the competitive ELISA and compared to those levels obtained from the same samples with a nephelometric assay. Although there was a significant correlation ($r=0.9393$, $p<00001$) between these two assay systems, serum albumin levels were found to be higher with the competitive ELISA. Because the expected normal values for human serum albumin greatly depends on the measuring system, it is recommended that each laboratory should establish its own normal range. In concordance with this, when the HSA (100 µg/ml) was measured with the nephelometry at CSF mode, it gave a 8.2 mg/dL result.

In summary, we describe a mAb which is specific for human serum albumin, and the utilization of this antibody to develop a method for identification and quantification of human albumin in samples. According to the data obtained from this study, it seems to be likely that the competitive ELISA can be used in diagnostic applications after some improvements.

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