Immunoaffinity layering enhances the sensitivity of antigen detection on nitrocellulose strips

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A simple strategy to remarkably increase the sensitivity of detection of antigens applied as dot or western blot on nitrocellulose membrane using human serum albumin as model antigen has been described. This involves subjecting the antigen bearing nitrocellulose strips to multiple incubation cycles with primary antibody and enzyme conjugated secondary antibody prior to staining for enzyme activity. The sensitivity of detection could be increased up to a thousand fold after three incubation cycles. Aggregation of human serum albumin could be detected by the multiple incubation procedure at very low protein concentration after electrophoresis and transfer onto nitrocellulose.

Introduction

Enzyme coupled immunoassays are widely used in revealing antigen antibody interaction on solid matrices such as nitrocellulose membrane in dot blot and western blot procedures, tissue sections, cell smear preparations as well as in ELISA.

Immunoassays and analogous assay systems are of increasing importance both for basic and applied research and for routine diagnostic purposes. Sensitivity is in most cases the key problem in immunological detection systems. In the past, there has been an explosive increase in the application of the technique in detection and quantitation of antigens/antibodies as well as in efforts to improve them.1,2 Interesting variations involving the use of biotinylated antibodies and avidin-conjugated enzymes have proved remarkably effective in improving the detection of dot blot analysis.

In efforts to raise the sensitivity of Clark electrode based glucose sensors, we successfully demonstrated that large amounts of the enzymes can be assembled on sepharose supports as alternate glucose oxidase/polyclonal antiguose oxidase IgG layers and the technique was described as immunoaffinity layering.3 In this paper, we show that a similar strategy can be used to remarkably increase the limits of detection of human serum albumin (HSA) on nitrocellulose in dot analysis as well as after transfer from polyacrylamide gels.

Materials and Methods

Materials

Human serum albumin, and Freund's complete and incomplete adjuvant were obtained from Scisco Research Laboratories, Mumbai, India. Genel Laboratories, Bangalore, India was the source of goat anti rabbit IgG- HRP conjugate. Nitrocellulose sheets and 4-chloro-1-naphthol were obtained from Sigma Chemical Co., USA. Pharmacia Biotech, Sweden was the source of Chelating Sepharose Fast Flow. The 96 well micro titer plates used were obtained from Greiner Labor-Technik, France.

Immunization

Rabbits weighing 2-3 kg were immunized by injecting them subcutaneously with 300 μg human serum albumin dissolved in 0.5 ml of 0.02 M sodium phosphate buffer (pH 7.5), mixed and emulsified with equal volume of Freund's complete adjuvant. After resting the animal for 15 days, three booster doses of 150 μg human serum albumin emulsified with the incomplete adjuvant were given at weekly intervals. The animals were bled and the serum isolated was decomplemented and stored at -20°C. Formation of
antibodies was monitored by immunodiffusion and ELISA as described⁵.

**Purification of IgG**

Rabbit IgG was isolated by a slight modification of the method of Fahey and Terry⁶. Rabbit blood was allowed to coagulate at 22°C for three hours and then centrifuged at 2,000 g for 10-15 min to isolate the serum. The 20-40 per cent ammonium sulphate fraction of the serum was dissolved in minimal amount of sodium phosphate buffer (20 mM, pH 7.2) to obtain the IgG which was dialyzed against the buffer extensively. The fraction was further purified by ion-exchange chromatography on a DEAE cellulose column⁶.

**Isolation of F(ab)²**

For the preparation of F(ab)², the IgG was incubated with pepsin (molar ratio of IgG: pepsin, 100:1) in 0.2 M acetate buffer (pH 4.5) for 20 hr at 37°C. The reaction was stopped by the addition of adequate amount of 1.0 M tris to pH 8.0 and dialyzed extensively against the buffer. Immobilized Metal ion Affinity Chromatography (IMAC) was performed by using the Fast Flow, Imino Diacetic Acid (IDA) Sepharose. The IDA matrix was washed with 50 mM EDTA and 500 mM NaCl (pH 8.0). CuCl₂ solution (20 mM) was mixed to load the matrix with Cu²⁺ ions. The matrix was then washed with the operating buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5) and mixed with the dialyzed extract. After incubation for one hour at room temperature, the unbound F(ab)² was collected by centrifugation at 1000 g⁷.

**Dot blot/Western blot**

For immunodetection of HSA dots, appropriate quantities of the protein dissolved in 0.02 M sodium phosphate buffer (pH 7.4) were applied on the nitrocellulose strips with the help of a micro syringe. The strips were blocked with 2% (w/v) solution of skimmed milk in the buffer for two hr, washed thoroughly with wash buffer (0.01 M sodium phosphate buffer containing 0.15M NaCl and 0.05% Tween-20) and incubated in Petri-dishes containing 0.1 mg/ml anti-HSA IgG at 37°C for one hr. The strips were again washed with the wash buffer and further incubated with the goat anti rabbit IgG-HRP for one hr at 37°C. After washing the strips thoroughly once again with wash buffer, they were stained by incubating with 4-chloro-1-naphthol-H₂O₂ reagent⁸. Where indicated, the unstained strips were reincubated with anti-HSA-IgG, washed and incubated once again with the goat anti-rabbit-IgG-HRP conjugate. The incubation cycles with primary antibody and secondary antibody enzyme conjugated were repeated as desired prior to staining for the bound HRP. HSA was subjected to polyacrylamide gel electrophoresis⁹ and electrophoretically transferred to nitrocellulose at 4°C in a Hoefer series TE transfer apparatus operating at 100V for 12 hr. Transfer buffer contained 20% (v/v) methanol, 192 mM glycine and 25 mM Tris. The bands were visualized by incubating the strips first with the primary antibody followed by washing and then with the conjugate for the desired number of times as described, before staining with the 4-chloro-1-naphthol-H₂O₂ reagent.

**Results**

Fig. 1 shows that quantity of HSA readily detectable by dot analysis using the anti-HSA-IgG and goat anti-rabbit IgG-HRP conjugate under standard conditions used was 22,600 pg, although the sample bearing 2260 pg of HSA was also visible as a very faint spot. The intensity of the HSA spots increased remarkably and step by step by further alternate incubations with anti-HSA-IgG and the conjugate, and those bearing far lower concentration of HSA, which were not visualized under standard conditions also became visible. As evident from Fig. 1, the HSA spot bearing 22.6 pg of the protein was also readily detectable after four incubation cycles with anti-HSA-IgG and the conjugate. While it was possible to further increase the sensitivity of the
procedure by increasing the number of the incubation cycles with anti-HSA IgG and the enzyme conjugated secondary antibody, this was accompanied by a marked increase in background staining.

In view of the high probability of the secondary antibody components of the conjugate recognizing the non-$F_c$ regions of the primary antibody, it was felt that removal of the $F_c$ portion of the latter may improve the binding process by decreasing steric hindrance. Enhancement in binding of the conjugate (on strips containing antigen with bound primary antibody) is expected to improve the sensitivity of antigen detection. Attempts were therefore made to investigate if the replacement of anti-HSA-IgG (primary antibody) with $F(ab)'_2$ derived thereof will enhance the sensitivity of detection of HSA. As shown in Fig. 2, the improvement in sensitivity as a result of substitution of $F(ab)'_2$ (strip A) for IgG (strip B) was not very high but appeared significant. The spot bearing even 11.3 pg HSA was clearly visible after four incubation cycles involving $F(ab)'_2$ and the intensities of the spots bearing higher concentrations of the protein increased as compared to those stained after layering using intact anti HSA IgG. Removal of the $F_c$ region may thus facilitate the enhanced binding of secondary antibody conjugate by making antigen binding domains on $F(ab)'_2$ region more accessible.

Fig. 3 shows the improvement in the detection limit of HSA in human serum samples by the multiple incubation procedure. As evident, HSA in the human serum sample bearing 39,440 pg of protein was barely detectable by the standard procedure. Successive alternate incubations caused, as described earlier, the appearance of new spots and marked intensification of those already visible. At the end of four incubation cycles, albumin in the human serum samples bearing 32 pg of protein was also detectable as a faint spot while that containing 39 pg protein was more intense.

Considering that albumin constitutes about half of proteins in plasma, the sensitivity of its detection in plasma samples appears comparable with that of purified HSA.

The usefulness of the multiple incubation procedure in detecting the well known HSA aggregation is shown in Fig. 4. As shown in panel Ia, aggregation is barely visible in the sample containing about 5.6 ng of HSA that migrated as a fast moving major band and a very faint band migrating about seventy five per cent of its distance, representing the aggregated form of the protein. The slow migrating band was visible somewhat more prominently in the sample bearing 56 ng, but was very clearly visible in that with 560 ng protein, which also showed the presence of an additional slower moving band. Successive layering with primary antibody and secondary antibody conjugate however resulted in visualization of additional bands and at the end of four incubation cycles, even the samples bearing 5.6 ng showed four additional slow migrating bands. Evidently even at the lowest concentration of HSA used, formation of aggregates takes place, but the aggregates were apparently present at concentration below the limits of detection of the standard assay. The aggregation could, however, be clearly visualized by the multiple incubation procedure, and five additional bands of various mobilities could be seen even in samples containing 5.6 ng of protein. Attempts to apply the multiple incubation strategy to ELISA of HSA by incubation with the primary and
Fig. 4—Immunoblot analysis of HSA [HSA was subjected to Polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Strips of panel I-IV were subjected respectively to single, two, three, four incubation cycles with antiHSA-IgG and goat anti rabbit IgG-HRP, respectively. Lanes a, b, and c contained 5.6 ng, 56 ng and 560 ng HSA respectively].

In most instances of infection, parasite specific antibodies are more easy to detect and hence used as diagnostic tools. However, diagnosis based on antigen may be more reliable although antigen concentrations present in blood even at the peak of infection period may be quite low. This together with the difficulty of obtaining adequate human biological samples for the assays, necessitates highly sensitive antigens detection procedures. The multiple incubation procedure described in this study may be very helpful in this respect. Another attractive feature of the procedure is its potential in increasing the sensitivity of antigen detection using commercial kits.

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References