Raising Monospecific Antibodies by Use of Protein Components Prestained with Remazol Brilliant Blue and Separated by Disc Electrophoresis on Polyacrylamide Gel


We have reported (Clin Chem 29: 42–44, 1983) that pre-staining with Remazol Brilliant Blue permits direct visualization of serum components on disc electrophoresis, and apparently purifies the proteins well. Here we have cut out the bands corresponding to the prestained albumin and transferrin after disc electrophoresis of normal human serum proteins, eluted some individual proteins into saline, and assessed their purity by immunoelectrophoresis and two-dimensional crossed immunoelectrophoresis against polyvalent antihuman serum. These two techniques indicated purity of these antigens. We inoculated rabbits with the eluates containing the pure antigens, and tested the resulting antibodies for monospecificity by immunoelectrophoresis, rocket electrophoresis, and single radial immunodiffusion. From the results we conclude that the antibodies raised against each component were monospecific, and that this is a simple, economical, rapid, and reliable method for obtaining a pure fraction of serum protein for use as an antigen.

Additional Keyphrases: albumin · transferrin · immuno-tests of purity

High-titer monospecific antibody is an essential prerequisite in many gel-precipitation reactions. Laboratories commonly produce their own antibody themselves by injecting rabbits with a pure antigen emulsified with complete Freund’s adjuvant. The main problem, however, is to obtain sufficiently pure antigen. The most common method for doing so is to separate a complex mixture of antigens by column chromatography and collect the individual components with a fraction collector. Another technique is ultracentrifugation. Some laboratories separate antigens by gel electrophoresis, staining a portion of the agar, agarose, or polyacrylamide gel to locate the particular protein, and eluting the corresponding section of an unstained gel with a suitable solvent to obtain a pure fraction. These procedures generally are time consuming, labor intensive, and also expensive because they require costly apparatus. Moreover, the fractions are often too much contaminated with other components.

We have previously reported pre-staining normal human serum with Remazol Brilliant Blue before polyacrylamide gel disc electrophoresis (1). Besides its advantages of speed and resolution, this technique can be used to purify proteins and other macromolecules. Unlike methods involving post-separation staining, it requires no prior fixation or subsequent destaining with acetic acid/methanol; hence the protein antigens are not denatured. We further showed (1) that the dye–protein complex eluted from individual pre-stained discs reacted with polyvalent antihuman serum, giving only a single well-defined arc in immunoelectrophoresis. We wondered if this material would retain its immunogenicity and thus be useful for raising monospecific antibodies. As a followup of the previous study, we here describe our findings: a simple method for raising antibodies against two prototype proteins, human serum albumin and transferrin, from their respective dye–protein complexes.

Materials and Methods

Reagents: Remazol Brilliant Blue R (C.I. no. 61200, cat. no. R-8001) was from Sigma Chemical Co., St. Louis, MO 63178. Acrylamide, N,N'-methylenebisacrylamide, and N,N,N',N'-tetramethylethylenediamine were from BDH Chemicals Ltd., Poole, U.K.

Procedures: We used 10 serum samples from apparently healthy adults in this study, pre-staining and subsequently separating the components as described previously (1). The various serum components were identified according to the nomenclature system suggested by Davis (2). The discs corresponding to albumin and the centrally placed transferrin were located, promptly cut before they diffused, pooled, and eluted with 2 mL of physiological saline. The gel pieces were crushed into fine granules and kept at 4 °C for 18 h to ensure maximum elution of the protein. We then centrifuged the eluate at 3000 × g for 10 min and tested the supernate for purity by immunoelectrophoresis and two-dimensional crossed immunoelectrophoresis against locally raised polyvalent antihuman serum. The presence of a single arc in immunoelectrophoresis and a single peak in two-dimensional crossed immunoelectrophoresis at the usual position with the expected shape and configuration was considered to indicate homogeneity (i.e., purity) of the antigen. Once the purity was ascertained, we used this fraction to immunize rabbits, mixing 1 mL of the eluate with 1 mL of complete Freund’s adjuvant and slowly inoculating the emulsified mixture subcutaneously at multiple sites. One month later, we administered a booster dose. Seven days later we bled the rabbits from the marginal ear vein.

The resulting antibody was tested for monospecificity by different immunological techniques. In immunoelectrophoresis, we charged the upper and lower troughs with the monospecific antibody under test and the polyvalent antihuman serum, respectively. We also tested the antibody by rocket electrophoresis after Laurell (3), and by the single radial immunodiffusion method of Mancini et al. (4), further to verify the monospecificity of the antibodies and to judge their ability to quantify the corresponding protein antigens.

Results

Immunoelectrophoretic separation of normal human serum revealed that the antibody against albumin was monospecific. It produced the characteristic boat shape at the anodic end, and the reaction of identity was evident by the upper and lower arcs’ typical fish-mouth configuration (Figure 1). Figure 2 shows single rockets with pointed tips and intense margins and of varying amplitudes proportional to
the concentration of albumin. Figure 3 shows that antibody against transferrin was also monospecific when assessed in immunoelectrophoresis of normal human serum. Figure 4 shows single rockets of different heights, confirming the monospecific nature of the antiserum against transferrin. Single and intense rings of immune precipitates with varying diameters were obtained in single radial immunodiffusion for both albumin and transferrin.

Discussion

Griffith (5) used Remazol Brilliant Blue, which is the potassium salt of the sulphato ethyl sulfone derivative of an anthraquinone sulfonic acid, for pre-staining individual proteins, then separated them in a horizontal polyacrylamide gel–sodium dodecyl sulfate system. The vinyl sulfone derivative of this dye, at an alkaline pH, binds covalently and stoichiometrically to primary and secondary amines and to alcohol and sulfhydryl groups, and can be used to detect as little as 3 \( \mu g \) of protein. We used the dye for the pre-staining and characterization of human serum proteins on disc electrophoresis (1).

Pre-staining permits direct visual location of the serum components. Sectioning of the individual bands is thus easy and contamination with other components less likely. The procedure is thus superior to analogous but blind procedures for purifying the proteins. The dye–protein complexes retain their ability to react with polyvalent antihuman serum. This suggested its immense potential in the production of monospecific antisera. This is particularly useful for us, because monospecific antisera are expensive and not easily available. This is a simple, cheap, less labor-intensive, fairly rapid, and reliable procedure for obtaining a pure protein and raising the corresponding antibody. The central aim here is not large-scale production but self sufficiency. Obviously, the quality of the primary resolution on disc electrophoresis is critical to antigen purity. The antibody of course can subsequently be made monospecific simply by adsorbing it with the eluate prepared from the contaminating fragments.

It would have been ideal to study the cross reactivity of antibody. For want of purified proteins we could not do this. Nevertheless, the antibody proved to be monospecific when reacted with normal serum containing several protein antigens. We have no data on the antibody binding capacity of the isolated antigen, as our main aim was production of a monospecific antibody, but we think that the binding capacity would not be different from conventional antigen–antibody union. This could be estimated either by optimal proportion techniques by using constant antibody optimum ratio or, still better, by estimating the antibody nitrogen of the precipitate. For lack of facilities we have not studied this facet.

The dye–protein complex we used for immunization served as a virtually pure antigen, because the dye itself is non-antigenic and non-toxic. We attempted production of antibody against two well-characterized components of serum, but any component, once characterized, could be used effectively to raise the particular antibody.
A New Endogenous Immunoenzyme Assay for Prostatic Acid Phosphatase Evaluated

Arthur L. Babson

I evaluated the performance of the "ORTH0 PAP-IA," an endogenous immunoenzyme assay for prostatic acid phosphatase. The procedure is based on double-antibody precipitation of the antigen, followed by quantification of its enzymic activity. The antibodies are in large excess, to speed the reactions and minimize sensitivity to variations in assay conditions. Enzymic activity is measured via an extremely sensitive colorimetric reaction, the analytical sensitivity of which exceeds that of the radioimmunoassay. Absorbance is linearly related to activity concentration up to an absorbance of 4.0, and only a single calibration standard is required. Within-run CV was <2%, between-run CV about 4%. Neither individual blanks nor assay in duplicate is required. In addition, the endogenous assay methods are stable solutions. Results correlated well with those by a standard radioimmunoassay (r = 0.993, n = 38).

Additional Keyphrases: reference interval • RIA compared • "kit" methods • prostatic cancer • enzyme activity

Measurement of acid phosphatase (EC 3.1.3.2) has been a routine laboratory procedure since its activity was first shown 45 years ago to be increased in the serum of patients with cancer of the prostate (1). Efforts to improve the specificity of the assay for the prostatic isoenzyme through the use of more specific substrates or inhibitors have not been entirely satisfactory. In addition, the enzymic assay procedures have lacked sensitivity and usually only identify advanced disease.

In 1975, Foti et al. (2) developed a radioimmunoassay (RIA) for prostatic acid phosphatase (PAP) that had significantly improved assay specificity and sensitivity. Since then, several commercial RIA kits and nonisotopic immunoassays for PAP have become available, stimulating renewed interest in this oldest tumor marker (3). The immunoenzyme assay evaluated here has excellent sensitivity and specificity, combined with several unique features not heretofore available in a commercial reagent kit: all components, including the standard, are stable solutions; reaction with antibody is complete in only 10 min; neither separate serum blanks nor duplicate assay tubes are required; absorbance is a linear function of PAP concentration over a wide range; and several alternative assay protocols are possible.

Materials and Methods

Materials

Reagents. The enzymoimmunoassay kits (Ortho PAP-IA) were supplied by Ortho Diagnostic Systems Inc., Raritan, NJ 08869. The RIA kits (EIAK) Prostatic Acid Phosphatase (125I) RIA kit were from New England Nuclear, North Billerica, MA 01862. Bovine serum albumin and p-nitrophenyl phosphate were from Sigma Chemical Co., St. Louis, MO 63178.

Serum specimens. Except where indicated, I used freshly drawn serum from ostensibly healthy men (Ortho employees). Abnormal activity concentrations of acid phosphatase were obtained by supplementing normal serum with concentrated preparations of acid phosphatase.

Acid phosphatase preparations. Prostatic acid phosphatase was obtained by dilution of seminal plasma. Acid phosphatase concentrates from platelets, leukocytes, and erythrocytes were prepared by isolating these cells from heparinized whole blood by repeated differential centrifugation, followed by repeated freezing and thawing of the platelet and leukocyte concentrates and lysis of the erythrocytes with distilled water. The lysed cell suspensions were centrifuged and the supernatant solutions used as sources of acid phosphatase.

Methods

RIA. I followed the directions in the New England Nuclear package insert, using the overnight incubation protocol for the first antibody reaction.

Immunoenzyme assay. I followed the directions in the Ortho package insert, except as otherwise noted. Briefly, the procedure is as follows: Incubate 200 µL of serum plus 40 µL (one drop) of rabbit antibody to human PAP for 10 min to complex all of the PAP in the sample. Precipitate the immune complex by adding 1 mL of goat antibody to rabbit IgG. Centrifuge the tubes, decant the supernates, and dissolve the precipitated immune complex (with PAP activity intact) in 0.5 mL of substrate: 4 mmol of a-naphthyl phosphate per liter of citrate buffer (0.1 mol/L, pH 5.6). After 30 min at 37 °C terminate the reaction by adding 0.5 mL of 0.5 mmol/L diazotized 5-nitro-2-aminoethylbenzene and then 1 mL of 0.3 mol/L NaOH. Determine PAP activity by comparing the absorbance at 620 nm with that of a PAP standard similarly treated.

One unit (U) of PAP activity corresponds to that amount...