Analysis of antigenic response and purification of anti-BSA specific γ - globulin fraction from ovines

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ABSTRACT

The serum protein electrophoresis test demonstrates a significant deviation from the normal gamma-globulin levels in different infectious states of the animal. The aim of this study was to evaluate the influence of BSA as antigen on total serum proteins and their fractions in ovines and raising antibodies against BSA. From each of the four animals considered for experimentation, pre immune as well as post immune blood samples were collected. For all serum samples, γ-globulin fraction concentrations were determined by Biuret method, after they were separated on Sephadex G -200. The gamma globulins were identified electrophoretically and the interaction of antigen and the antibody raised was confirmed by Ouchterlony double immuodiffusion method after fractions of sheep serum were obtained by gel filtration on Sephadex G-200. The purification of the IgG-2 fraction was performed while passing the purified fraction in DEAE (Diethylaminoethyl), A-50 column. The study revealed an increasing pattern in the total protein concentrations in general and appreciable increase in the gamma fraction in particular. Both the modules showed 95% confidence interval with students paired t-test.

Keywords: Serum proteins, Antibody Purification, Gamma fraction

Blood proteins comprise the major portion of the immune system and are separated into fractions of five distinct classes: albumin, α_1-globulins, α_2-globulins, β-globulins, and β-globulins (immunoglobulin's). The gamma-globulin fraction contains the immunoglobulins, a family of proteins that function as antibodies. Antibodies, in response to infection, allergic reactions, and organ transplants, recognize and bind foreign bodies or antigens, to facilitate their destruction by the immune system. The immune response is regulated by a large number of antigen-specific gamma-globulins that fall into five main classes called IgG, IgA, IgM, IgD and IgE. The serum protein electrophoresis test demonstrates a significant deviation from the normal gamma-globulin levels in different infectious states of the animal. The objectives underlying this study were to evaluate the deviation in protein concentrations of the serum post infection along with raising and purifying anti-BSA specific antibodies from serum.

MATERIALS AND METHODS

Serum collection and antigen preparation

In all experiments, ram (n=4) of mixed breed in the age group of 1 ½ - 2 ½ years were used. They were clinically healthy during the entire experiment. Preimmune blood (10 ml) was collected from each of the four sheep, using sterile syringes with due care against haemolysis and transferred to vacutainers appropriately tagged and were let to clot. The serum was collected from each of the tube and transferred to appropriately tagged cryovials and stored at - 80^{o}C till further processing. The sera were assayed for total protein concentrations by Biuret method as will be compared for the changes post administration. 1 ml antigen BSA (0.5mg/ml sterile H_2O) in Freund's complete adjuvant (1 ml) was administered subcutaneously at multiple sites, in each of the four animals. The blood (10 ml) was collected after 21 days and same day the booster dosage of antigen (1 ml) BSA (0.5 mg/1ml sterile H_2O) in (1ml) Freund's incomplete adjuvant was administered. Both pre as well as post administration sera were first directly assayed for protein measurements for total protein concentrations. From among the above pre-immune sera's, one was taken as a reference for assessing the deviations post-infection, by electrophoretically running it against the post-infection sera from all the four animals on 12.5% SDS-PAGE. (Figure 1)

Serum Protein Precipitation

This stepwise procedure for the fractionation of serum proteins was done according to Hebert *et al*. (1973) A stock solution of saturated ammonium sulphate (SAS) was prepared and stored at room temperature (approximately 25°C). Working solutions of 70, 80, and 90% SAS were prepared (v/v) fresh as needed from the stock saturated solution. Equal volumes of these solutions were mixed with various antisera to prepare mixtures of 35, 40, 45% SAS. 6ml of all the pre and post administered sera's was gently stirred while an equal volume of an ammonium sulphate solution to the final concentration of 45%, was slowly added and mixed well. The reaction mixtures were set aside at room temperature for 4 hours and then centrifuged to pack the precipitated protein. The supernatant fluid was removed and stored for later analysis. The precipitate was resuspended and dissolved in distilled water to a final volume equal to the original volume of serum. For a second precipitation, the dissolved proteins were gently stirred while an equal volume of an ammonium sulphate solution to the final concentration of 40% was slowly added. The mixture was immediately centrifuged to pack the formed precipitate, and the supernatant fluid was discarded. The precipitate was dissolved and brought to volume as before. A third precipitation was handled in the same manner, but the ammonium sulphate concentration was reduced to 35% in the final volume, which leaves behind less than 1 % of the albumin. All fractions were then dialyzed against frequent changes of pH 8, 0.85% NaCl solution until sulphate was no longer detected in the dialysate. The saline was brought to pH 8.0 with a few drops of 10% NaOH. A

small volume of saturated barium chloride solution was added to an equal volume of all well-mixed saline dialysates to check for the presence of sulphate. Since no cloudiness resulted, the dialyzed fraction was considered substantially free of sulphate. Protein concentrations were measured by the Biuret method using spectrophotometer.

γ – Globulin Fractionation

The Protein solution from above dialysates (0.5 ml) from each animal was loaded in 200 gm packed Sephadex DEAE G-200 (prewashed) column with a void volume (V_{vol}) of 45.2 ml (determined by Bromophenol Blue dye) and the fractions were collected each after 1 ml in different tubes with 20mM phosphate buffer, pH 7.2 and each fraction was checked for presence of protein. Thus the total fractions collected resulted in the sequence of their molecular weights with albumin coming first and gamma fraction coming subsequently. This second fraction was quantified against each of their respective pre-immune gamma fractions showed increase in the protein concentration by (mean ± S.D) 1.5 ±0.452g/dl. (Table 2) The students paired t-test was run on this data showed a confidence interval of 95% ranging from 0.7313 -2.1937. The stastical analysis revealed that both the total protein concentrations as well as ã- fraction concentrations were increased significantly with 95% confidence interval when data was analysed by paired students t-test.

Table 2: Comparison of globulins-fraction concentrations pre and post-administration of BSA.

Protein Conc. (mg/ml)	n 1	n 2	n 3	n 4
Pre-administration	18.87	19.04	19.9	18.5
Post-administration	20.89	19.97	21.45	19.85

Isolation of purified antibodies

Anti-BSA antibodies were isolated with an immunoadsorbent consists of bovine serum albumin cross linked with glutaraldehyde on nitrocellulose membrane (Avrameas and Ternynck, 1969). Coloumn was washed five times with PBS, until the absorbance A_{280} of the washing fluids became less than 0.05. The anti-BSA antibodies were eluted from the immunoadsorbent with 0.1N glycine-HCl buffer pH 2.8 and immediately adjusted to pH 7.6. The IgG1 and IgG2 antibodies were separated by chromatography on DEAE-Sephadex A-50. When a purified IgG fraction from anti-BSA sheep sera (from four animals) was chromatographed on DEAE-Sephadex A-50, two peaks were obtained. The first peak eluted with the equilibration buffer contained the fraction which showed high reactivity with BSA and concluded to be IgG2 subclass whereas the second, which was obtained after the 500 mM NaCl gradient was applied, contained the IgG1 subclass.

RESULTS AND DISCUSSION

The electrophoretic profile on 12.5% SDS-PAGE pre and post-infection revealed adequate increase in all the protein fractions and immense increase in gamma fraction in particular.

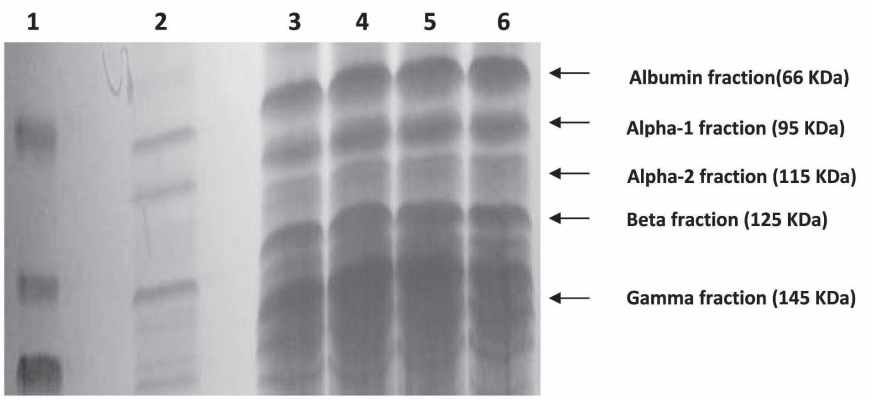

Figure 1: Electrophoretic Profile of serum proteins lane 1 representing protein ladder lane 2: pre-infection serum, lane 3,4,5,6 : sera from n1,n2,n3,n4 respectively

The above IgG2 fractions from all the animals (n=4) were analysed for their activity by setting affinity reactions by Ouchterlony passive double immuodiffusion. (Figure 2).The plates (agarose in Barbital buffer-Barbiturate-sodium barbiturate buffer, pH 8.6, 75mmol/L) were prepared and the cork borer was used to carve the wells at equidistant corners of the square with a central well. The IgG2 containing wells showed immunoaffinity lines towards the central well containing BSA, when incubated at 37°C for 48 hrs, confirming the anti-BSA specific antibodies and their affinity towards the BSA.

The study shows increase in total protein concentrations (Table 1) in all animals in general with the confidence interval of 95% ranging from 4.277-10.973, and the substantial increase in the concentrations in ã – fraction in particular. (Table 2) which is consistent with the concept underlying the immune response that even an antigen being narrowly different from self substances shows specific and strong response to foreign substances, as was done in this study where BSA (as antigen) being narrowly different from the albumin from ovines.

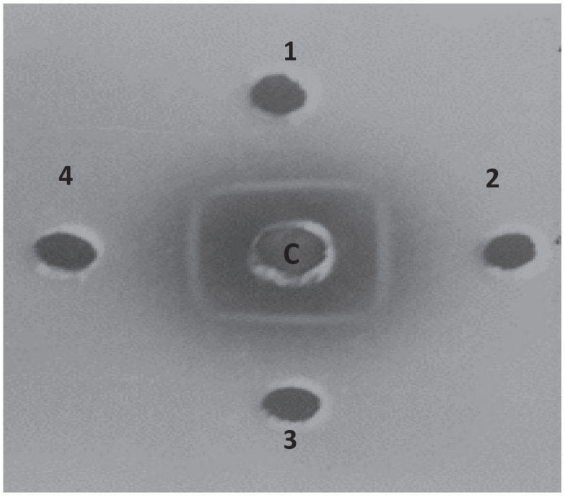

Figure 2. Ouchterlony immuodiffusion: wells 1, 2, 3, 4 showing antibody affinity towards central well (c) containing BSA (antigen) on agarose-barbital plates, after 48 hrs of incubation at 37ºC.

The study concurs the results of Tiselius *et al.* (1939) showing an increase in the gamma fraction after antigenic challenge. The purification procedure described here is a collective procedure, otherwise to the enzymatic procedures for the fractionation of the specific antibodies from the gamma fraction as reported by Nisonoff *et al.* (1960) and Porter *et al.* (1972) and their purification by ammonium sulphate as described by Herbert et al (1969).

Table 2: Comparison of globulins-fraction concentrations pre and post-administration of BSA.

Protein Conc. (mg/ml)	n 1	n 2	n 3	n 4
Pre-administration	18.87	19.04	19.9	18.5
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CONCLUSION

The study resulted in raising the anti-BSA specific antibodies in ovines, which showed strong affinity towards the antigen on Ouchterlony immuodiffusion plates, revealing the specificity of the immune system and generalising the procedure for the purification of antibodies against any specific antigen.

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