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B-serum is Highly Immunogenic when Compared to C-serum Using Enzyme Immunoassays

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Two enzyme immunoassays were utilized to identify the source of antigens eluted from natural rubber latex gloves. Both the indirect enzyme-linked immunosorbent assay (ELISA) and a nitrocellulose based dot enzyme immunoassay showed that a rabbit antiserum directed against proteins eluted from gloves was more reactive against B-serum than against C- serum. It was further shown that the dose response relationship of C-serum and rubber particle extract to rabbit anti-glove protein was similar. When rats were immunised with C-serum, an indirect ELISA showed that this serum was more reactive with B-serum antigens than with C-serum, suggesting that B-serum is likely to be more immunogenic than C-serum.

The concern about the increase in numbers of users presenting with Type I hypersensitivity reactions to products manufactured from natural rubber latex (for reviews, see for example, Tomazic, *et al.*,[1] and Hamann[2]) has highlighted the need to understand the nature and source of antigens and allergens found associated with these products. Specific identification of the allergens eliciting IgE responses in patients has not been achieved, although a number of proteins and polypeptides have been identified by reference to molecular mass.[1,2]

In the absence of specific identification of allergenic proteins, it may be useful from a practical point of view, to identify the main fraction(s) of natural rubber latex which may be the source of antigenic material found in latex products. Sunderasan and Yeang[3] have reported that using two-dimensional immunoelectrophoresis and Western blotting techniques, latex bottom fraction appears to be a major source of immunogenic glove proteins. While immunoprecipitation-in-gel techniques and Western blotting may show convincingly some qualitative aspects of the antigens being studied, such as the number of polypeptides in a mixture and their apparent molecular masses, there are, nevertheless, some major limitations to these techniques, not least being low sensitivity. Western blotting is further disadvantaged by the fact that under the conditions used in sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE), the antigenicity of many proteins and polypeptides may be destroyed because conformational epitopes are normally lost.

This report describes the use of two different enzyme immunoassays to determine the reactivity of proteins from various fractions of centrifuged natural rubber latex. As

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described by Moir[4], when latex is centrifuged, it separates into three layers with the rubber cream on top, the 'bottom fraction' containing lutoids at the bottom and the C-serum in between. When the 'bottom fraction' is subjected to repeated freeze-thaw cycles and recentrifuged, the supernatant collected is the B-serum. Tata[5] has reported that natural rubber latex contains about 1% proteins, with approximately 26% of these being associated with rubber cream, 46% in C-serum and 28% in the 'bottom fraction'. Most of the proteins of the rubber particles are membrane proteins and are normally insoluble, although in the presence of ammonia which is often added as a preservative, some of these proteins may become solubilised.

MATERIALS AND METHODS

Latex Proteins

Standard methods for the collection and preparation of B-serum and C-serum have previously been described[3]. Proteins associated with the rubber particles in rubber cream were extracted using 0.05% Triton X-100 and 0.5% SDS, vortexed and centrifuged. The supernatants recovered contained the rubber particle proteins.

Rabbit Antiserum Directed Against Glove Proteins

Glove protein eluates were prepared as described previously[3] from non-sterile examination gloves purchased from seven different manufacturers and pooled to be used as the immunogen. Rabbits were immunised subcutaneously with 0.4 mg glove eluate in complete Freunds adjuvant followed by seven booster doses in incomplete Freunds adjuvant at two week intervals as described previously[3]. Serum obtained was frozen in aliquots at –30°C until use.

Rat Antiserum Directed against C-serum

A total of nine Sprague Dawley rats were intravenously immunised through the tail vein with 4 mg of C-serum diluted in phosphate buffered saline followed by a second dose nine days later. The rats were bled by cardiac puncture ten days after the booster dose and serum separated. Two unimmunised rats were also bled by cardiac puncture to provide a source of non-immune rat serum. All sera were stored in aliquots at –30°C.

Dot Enzyme Immunoassay

The method used was an adaptation of the dot enzyme immunoassay (EIA) designed for determination of antibodies to dengue virus antigens[6,7] and is described briefly here. Nitrocellulose membranes (0.45 µm pore size) were dotted with 2 µl drops of B-serum and C-serum dilutions in phosphate buffered saline, pH 7.4 (PBS). These were allowed to air dry and free protein binding sites were blocked by soaking for 30 min in a blocking buffer containing 5% non-fat skimmed milk in PBS (PBS-SM). The membranes were rinsed in 2 changes of distilled water and allowed to air dry before storing at 4°C until use.

Antigen coated membranes were placed into a minimum volume of rabbit antiserum dilutions in PBS-SM and incubated at room temperature with rocking. After incubation for 60 min, the unbound antisera was removed using a washing protocol of three washing cycles using PBS with a 5 min soak time per cycle on a rocking platform. After the last wash, swine anti rabbit Ig – HRP (Dakopatts, Denmark) at a dilution of 1:2000 in PBS-SM was added and incubated for 60 min in the dark at room temperature with rocking. The unbound conjugate was removed by washing as

described above and colour development was achieved by incubating in hydrogen peroxide/ 4-Chloro-1-Naphthol for 30 min in the dark. The reaction was stopped by rinsing the membrane in distilled water.

Indirect ELISA

Standard published methods were used for optimising the ELISA[8-10]. Flat bottomed (Costar, USA) microtitre plates were coated using 100 µl of B-serum and C-serum dilutions in carbonate-bicarbonate buffer, pH 9.6. Proteins were allowed to bind overnight at 4°C and the following day the plates were blocked for 2 h using a solution containing 0.5% casein in PBS. The plates were then washed twice with a 1 min soak time between washes. The wash buffer used was PBS containing 0.05% Tween-20 (PBS-T).

Serial dilutions of rabbit antiserum were made in PBS containing 1% foetal bovine serum (FBS). 100 µl were loaded into the wells of the antigen coated microtitre plate and incubated for 1 h at room temperature after which unbound antisera was removed using the same washing protocol described above. The conjugate, swine anti-rabbit Ig – HRP (Dakopatts, Denmark), was diluted 1:2000 in PBS containing 1% FBS and 100 µl dispensed into each well. Where rat antiserum was used, the conjugate was goat anti-rat IgG - HRP (KPL, USA). After incubation in the dark for 1 h at room tem- perature, the plate was washed as described above and 100 µl of a substrate solution was added (hydrogen peroxide-OPD). Colour was allowed to develop for 30 min before the reaction was stopped with 50 µl of 2.5*M* sulphuric acid. Absorbance was read using a spectrophotometric plate reader (Anthos 2001, Austria) with a dual wavelength mode at 492 nm reading wavelength and 620 nm as reference.

RESULTS

Figure 1 shows a checkerboard titration of rabbit anti-glove proteins against B-serum and C-serum in a tenfold dilution series. The B-serum series in the top row and the C-serum series in the bottom row of each piece of nitrocellulose begin with a 1:10 dilution and a 1:5 dilution respectively. Assuming that B-serum contains approximately 24 mg protein per ml and C-serum contains approximately 12 mg protein per ml, the starting protein concentrations in both series are approximately equivalent (2.4 mg/ml). It is clear from this

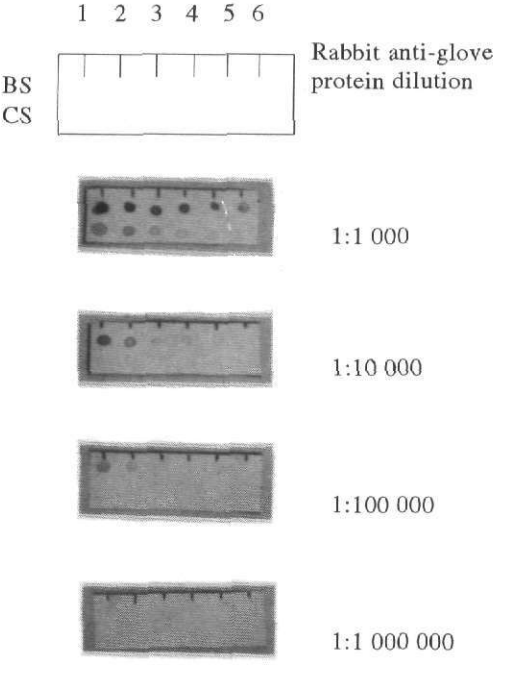

Figure 1. Checkerboard titration of rabbit antiserum against glove protein eluates by DEIA using B-serum and C-serum dilutions. Concentration of protein dotted was as follows: (1) 2.4 mg/ml (2) 0.24 mg/ml (3) 24 µg/ml (4) 2.4 µg/ml (5) 0.24 µg/ml (6) 0.024 µg/ml.

DEIA checkerboard that at all dilutions of rabbit anti-glove protein tested, B-serum is more readily picked up by the antisera. When rabbit anti-glove protein was used at 1:1000 dilution, C-serum titred out to 0.24 mg/ml while B-serum was still strongly positive at a concentration of 24 µg/ml. In the next panel antiserum at a dilution of 1:10,000 is still able to detect B-serum with an endpoint titre of 0.24 mg/ml while C-serum is barely detectable.

In this same checkerboard titration the endpoint titre of the rabbit anti-glove protein can be determined to be 1:10000 against C-serum and 1:1 000 000 against B-serum.

A similar titration of rabbit anti-glove protein was carried out using the indirect ELISA technique and the results are shown in *Figure 2* where a single dilution of B-serum and C-serum was coated onto microtitre plates and the rabbit antiserum titrated out in a two-fold dilution series. It is clear from the histogram shown that the rabbit glove protein titred out to about 1:16 000 against C-serum while the titre against B-serum was clearly greater than 1:64 000.

In a series of dose response experiments using a single dilution of rabbit anti-glove protein (1:1000) and ten-fold serial dilutions

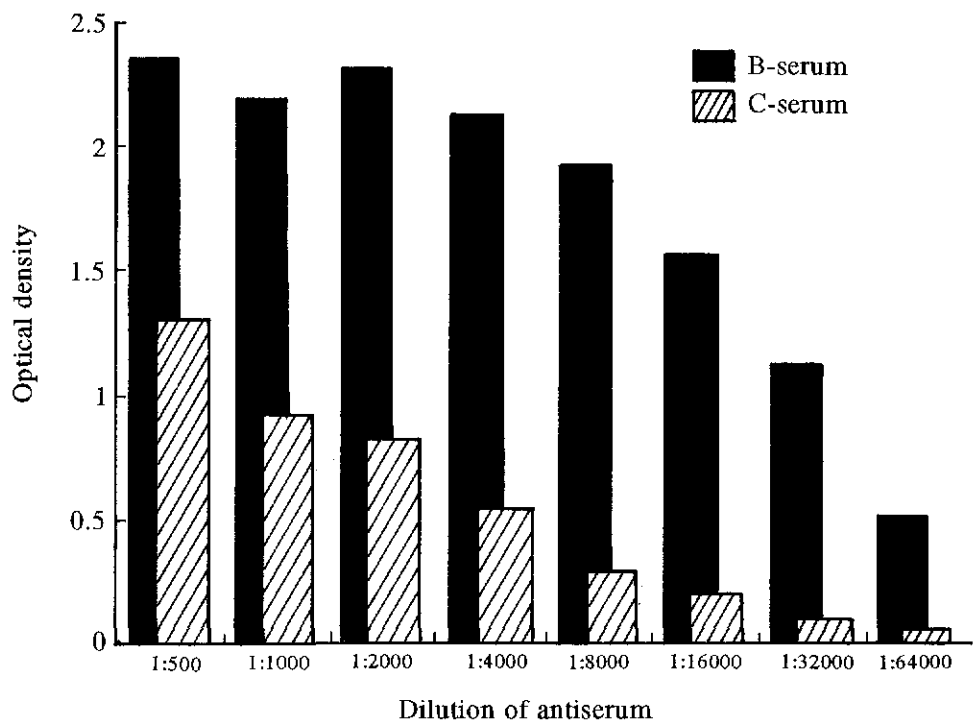

Figure 2. Titration of rabbit antiserum against glove proteins in an indirect ELISA using microtitre plates coated with B-serum or C-serum at a single antigen dilution of 1:1000.

of B-serum and C-serum in an indirect ELISA, it was determined that the rabbit antiserum always reacted more strongly with B-serum than with C-serum. *Table 1* summarises this data showing the ratio of the optical density of the peak antiserum reactivity to B-serum over the peak antiserum reactivity to C-serum for four separate experiments.

Figure 3 illustrates a typical dose response relationship of B-serum, C-serum and rubber particle extract. The B-serum dose response curve clearly shows a stronger reactivity against rabbit anti-glove protein than either C-serum or rubber particle extract which have relatively similar levels of reactivity.

TABLE 1. COMPARATIVE REACTIVITY OF B-SERUM AND C-SERUM WHEN TESTED AGAINST RABBIT ANTI-GLOVE PROTEIN

Experiment	Ratio $OD_{(BS)}/OD_{(CS)}$
1	1.48
2	1.75
3	1.83
4	1.57

To ask the question whether C-serum is simply not a potent immunogen, we immunised rats with C-serum and *Figure 4* shows the

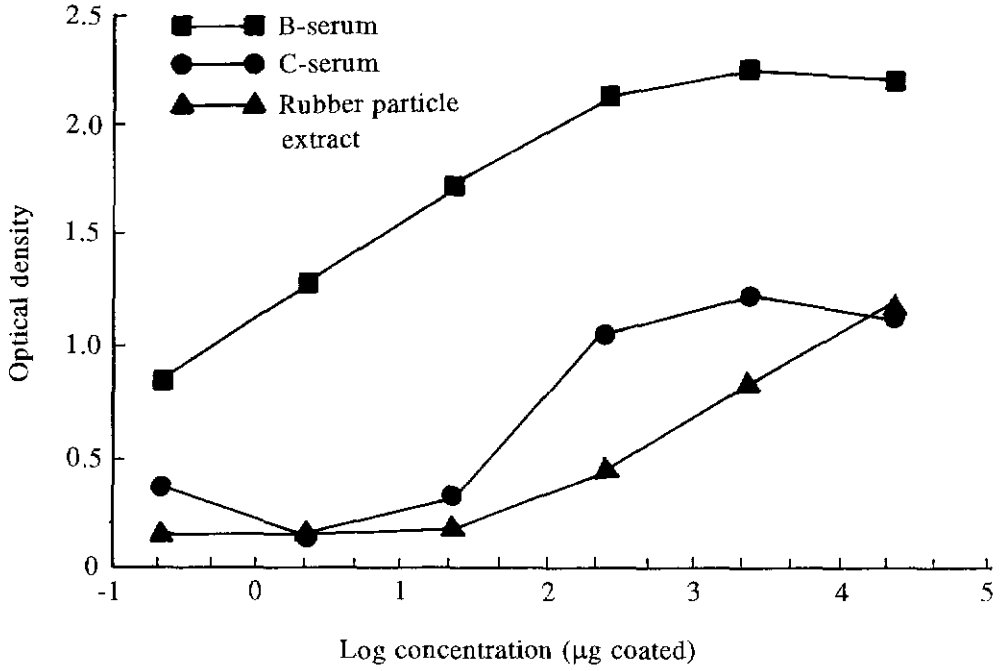

Figure 3. Dose response relationships of B-serum, C-serum and rubber particle extract in an indirect ELISA using rabbit antiserum against glove proteins at a single antiserum dilution of 1:1000.

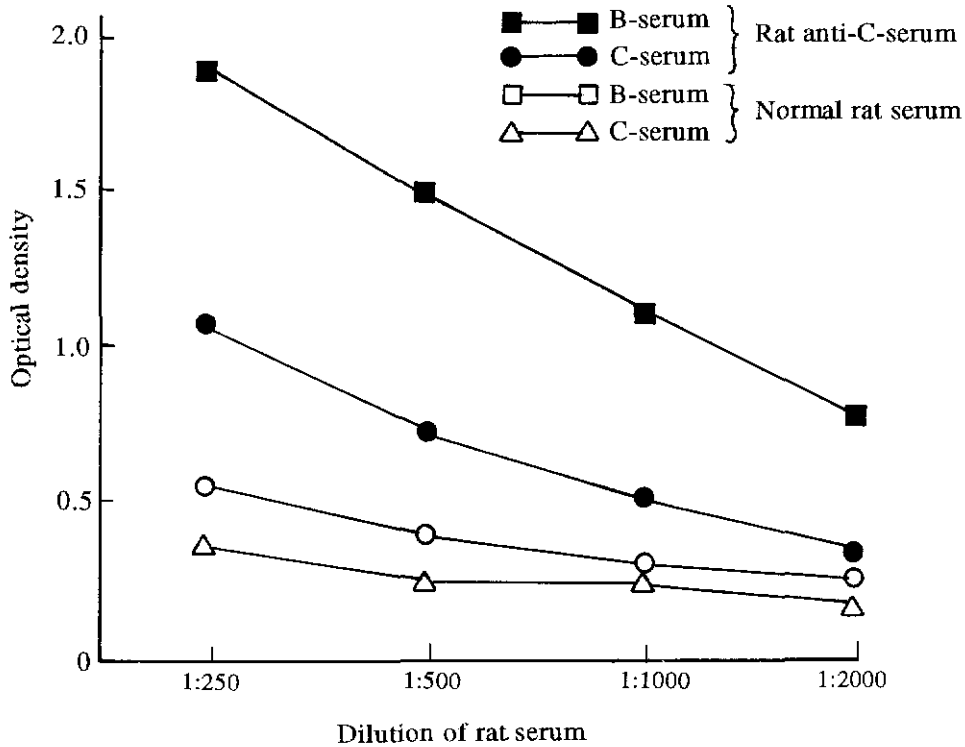

Figure 4. Indirect ELISA showing the reactivity of B-serum and C-serum when measured using a rat antiserum prepared against C-serum as immunogen. The ELISA wells were coated with 2.4 μg B-serum or C-serum and reacted with two fold serial dilutions of rat antiserum against C-serum or normal rat serum.

titres of this antiserum against B-serum and C-serum by indirect ELISA. Clearly the reactivity of the rat anti-C-serum was stronger against B-serum than against C-serum at all dilutions of antiserum tested. The total protein coated was approximately 2.4 mg per well for both B- as well as C-serum thus confirming that there must be B-serum proteins which are highly immunogenic. Only a very minor fraction of the proteins in C-serum would be from B-serum which would unavoidably contaminate C-serum due to ruptured lutoids in the process of collection and centrifugation of fresh non-ammoniated latex. The serum prepared from non-immune mice did not react appreciably with either B-serum or C-serum although the background optical densities were clearly higher for C-serum than B-serum.

CONCLUSION

All the data described above clearly indicate that the rabbit antiserum prepared against glove protein eluates reacts more strongly with B-serum than with C-serum as previously reported by Sunderasan and Yeang[3]. This report confirms using enzyme immunoassays, that the glove protein eluates contributing to the immunisation of the rabbits derive mainly from B-serum although there are clearly C-serum proteins eluting from

the latex gloves as well. This could be so for one or both of two reasons. The first possibility is that it might simply be that more B-serum than C-serum proteins elute from latex gloves, implying that more soluble B-serum proteins are retained in the gloves during the manufacturing process. The second possibility is that of the proteins eluting from the gloves, those deriving from B-serum are far more immunogenic than those from C-serum. The indirect ELISA using rat antiserum raised against C-serum has shown that there are indeed B-serum proteins which are highly immunogenic, to the point that small amounts of contaminating B-serum in the C-serum preparation stimulates a stronger immune response to B-serum than to C-serum in rats immunised with C-serum. This, however, does not exclude the possibility that besides the fact of B-serum being more immunogenic, there may also be more B-serum than C-serum proteins eluting from gloves.

This study shows that both the dot EIA and the ELISA confirm earlier observations that B-serum contributes more immunogenic polypeptides to the soluble proteins elutable from natural rubber latex gloves than does C-serum.

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