Cross-reaction between Streptococcal Protoplast Membrane and Human Glomerular Basement Membrane*

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SUMMARY A cross-reaction between streptococcal protoplast membranes (SPM) and human glomerular basement membranes (GBM) was investigated by countercurrent-immunoelectrophoresis (CIE) and enzyme-linked immunosorbent assay (ELISA) techniques. A cross-reaction could not be detected with CIE; however, cross-reactivity was detected by ELISA. The latter was carried out either with a collagenase digest of GBM and rabbit antisera specific to protoplast membranes of *Streptococcus pyogenes* [both nephritogenic strains (M types 12 and 57) and non-nephritogenic strains (M types 6, 18 and 19)], or with SPM antigen and rabbit anti-GBM antisera. In addition, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of SPM and GBM revealed one polypetide band, which appeared to be common to GBM and all types of SPM studied; the molecular weight of the band was approximately 60,000 daltons.

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Group A streptococcal protoplast membranes (SPM) are believed to be involved in the pathogenesis of post-streptococcal sequelae by one or more mechanisms.[1-7] While many investigators have agreed that the cross-reaction between SPM and human heart tissue may be the mechanism attributed to the pathogenesis of rheumatic fever,[2,4,7] the role of SPM in the pathogenesis of post-streptococcal glomerulonephritis (PSGN) is still controversial. A cross-reaction between SPM and human glomerular basement membrane (GBM) has been reported by many investigators[1,5,6] and has been suggested as being the mechanism involved.[1] However, some investigators have failed to confirm this cross-reaction[3] and have suggested that SPM may be involved in the pathogenesis of PSGN by forming immune complexes, which are deposited in the glomeruli.[3]

Since there is such disagreement about the role of SPM in the pathogenesis of PSGN, attempts have been made to clarify further the matter. Immunochemical techniques and ELISA were selected as tools for this investigation. Unlike other experiments mentioned above, our investigations were carried out with protoplast membranes obtained from both nephritogenic and non-nephritogenic strains of *Streptococcus pyogenes.*

MATERIALS AND METHODS

Streptococcal protoplast membrane antigens

The protoplast membranes were prepared from three non-nephritogenic strains and two nephritogenic strains of *Streptococcus pyogenes*, by a phage-lysin technique.[8] The three non-nephritogenic strains were M types 6 (NCTC 8302), 18 (NCTC 8320) and 19 (NCTC 8194) and the two nephritogenic strains were M types 12 (NCTC 10085) and 57 (NCTC 100190). All bacterial strains were supplied as lyophilized cultures by the Streptococcus Reference Laboratory, Central Public Health Laboratory, Colindale, London.

Solubilised SPM antigens were triton X-100 extracts and sodium deoxycholate extracts of each type of SPM. The triton X-100 solution

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was 4% (v/v) in 0.05 M tris HCl, pH 8.6. The sodium deoxycholate solution was used at a concentration of 5 mg/ml. Both extractions were carried out at 23°C for two hours and the concentration of SPM in both extracting detergents was 5 mg/ml. Both preparations of solubilised SPM were stored at -20°C in 0.2 ml aliquots.

Human glomerular basement membrane antigen

Glomerular basement membranes were prepared from human cadaver kidneys.[9] The soluble antigen was prepared from lyophilised GBM by the method described previously.[10] Briefly, the lyophilised GBM was suspended in 0.1 M tris-acetate buffer, pH 7.4, containing 0.005 M calcium acetate, to a concentration of 25 mg/ml. Digestion with collagenase was carried out at 37°C for 48 hours. The mixture was then centrifuged at 3,000 x g for 15 minutes and the soluble collagenase digest (CD) was stored in 0.1 ml aliquots at -20°C.

Rabbit anti-streptococcal protoplast membrane antisera

Antisera to SPM 6, 12, 18, 19 and 57 were raised by using the following immunisation procedure in rabbits. Three milligrams of SPM were administered on days 1, 7, 14, 21 and 28. On days 1 and 7, the antigen was suspended in Freund's Complete Adjuvant (FCA) containing 2% Tween 80 in 0.15 M NaCl and both intramuscular (i.m.) and subcutaneous (s.c.) injections were given. On the remaining days, only subcutaneous injections were administered, using FCA on day 14 and Freund's Incomplete Adjuvant (FIA) on days 21 and 28. A booster injection of 3 mg of SPM in FIA was given subcutaneously on day 65.

All rabbits were bled from the marginal ear vein before immunisations and on days 42, 55, 63, 79, 84 and 86 throughout the immunisation course. The sera obtained were stored at -20°C.

Rabbit anti-human glomerular basement membrane antisera

Rabbit anti-GBM antisera were raised by giving five weekly intramuscular injections of GBM in FCA, followed by a booster injection of GBM in normal saline, 85 days after the first injection. The animals were bled nine days after the booster injection.

Conjugate used in ELISA methods

The conjugate used was goat anti-rabbit IgG-alkaline phosphatase conjugate, which was purchased from Sigma London Chemical Co., Ltd., Poole, Dorset. This was used at the recommended dilution of 1:1,000.

Conjugate substrate

The conjugate substrate was p-nitrophenylphosphate (NPP), which was obtained in tablet form from Sigma London Chemical Co., Ltd. This was freshly prepared and was used at a concentration of 1 mg/ml in 0.05 M carbonate buffer, pH 9.8, containing 0.005 M $MgCl_2.6H_2O$.

ELISA for anti-streptococcal protoplast membrane antibodies

The ELISA method for the determination of anti-SPM antibodies was essentially the same as described previously.[11] Briefly, the SPM antigen used for coating disposable polystyrene tubes (Sterilin Ltd., Middlesex) was a sodium deoxycholate extract of each M type of SPM, at a protein concentration of 4 μg/ml. Antigen coating was carried out at 37°C for three hours. Serum and conjugate incubation was at 25°C for three hours and overnight respectively. The reaction of the conjugate and substrate was stopped with a 1.0 M sodium hydroxide solution. The colour which developed was read spectrophotometrically at 400 nm with a SP 500 Series 2 spectrophotometer (Pye Unicam Ltd., Cambridge) in 10 x 4 x 45 mm plastic cuvettes (Sarstedt (U.K.), Leicester).

ELISA for circulating anti-human glomerular basement membrane antibodies

This ELISA method was essentially the same as previously described.[10] In brief, CD, at a protein concentration of 10 μg/ml, was used for coating polystyrene tubes at 37°C for three hours. The incubation conditions for sera and conjugate were at 25°C for five hours and overnight respectively. A 1 M sodium hydroxide solution was used to stop the reaction of conjugate and substrate and the colour which developed was read spectrophotometrically, as above.

Countercurrent immunoelectrophoresis

Countercurrent immunoelectrophoresis was carried out with 7 μl of triton X-100 extracts of each M type of SPM, CD and each of the rabbit anti-SPM antisera. The gel used was 1% agarose type II (Sigma London Chemical Co. Ltd.) in barbitone buffer, pH 8.6, ionic strength 0.05, which was allowed to set at room temperature on the hydrophilic side of gel bond film (Miles Laboratories Ltd. Bucks). Wells for antigens and antisera were 3 mm in diameter and 3 mm apart. Electrophoresis was carried out at 150 V for 90 minutes, using barbitone buffer, pH 8.6, ionic strength 0.1 as the electrode buffer. The direction of current was from the antigen well towards the antiserum well. After washing in phosphate buffered saline (pH 7.1) and drying under soaked filter paper with a blow dryer, the gel was stained with 0.6% naphthalene black 12 B in 7% (v/v) acetic acid for five minutes. It was then destained with 7% (v/v) acetic acid, until the background was clear.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

The protoplast membranes of *Streptococcus pyogenes* M types 6, 12, 18, 19 and 57 and the collagenase digest of human glomerular

basement membrane were subjected to electrophoresis in a 14% polyacrylamide slab gel in the presence of sodium dodecyl sulphate, with the buffer system originally described by Laemmli.[12] Mixtures of various proteins with molecular weights ranging from 76-78,000 to 12,300 daltons (BDH Chemicals Ltd., Poole, Dorset) were used as standard markers. Electrophoresis was carried out in a PROTEAN Dual Slab Cell (Bio-Rad Laboratories Ltd., Hertfordshire), with a constant current of 50 mA, until the front dye reached the bottom of the gel.

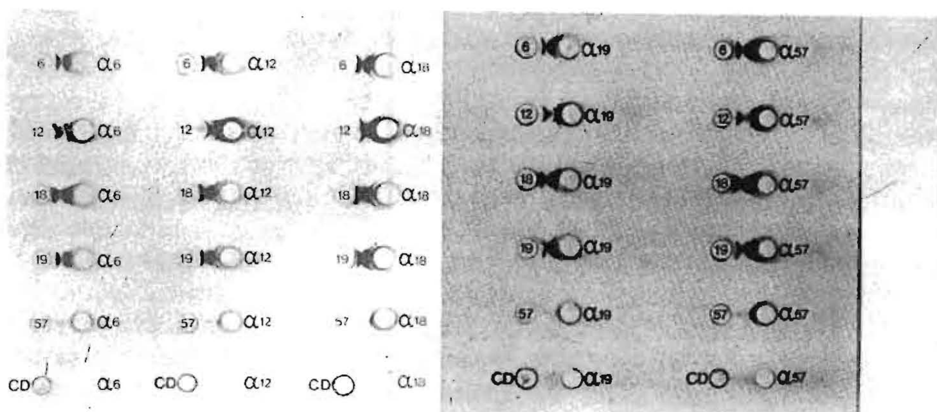

Fig. 1a &b Countercurrent immunoelectrophoresis of triton X-100 extracts of streptococcal protoplast membranes (SPM) (M types 6, 12, 18, 19 and 57) and a collagenase digest of human glomerular basement membrane (CD) against either homologous or heterologous rabbit anti-SPM antisera, obtained on day 55 (α 18 and α 19) and 86 (α 6, α 12 and α 57).

RESULTS

Countercurrent immunoelectrophoresis

The results of the countercurrent immunoelectrophoresis of triton X-100 extracts of protoplast membranes obtained from *Streptococcus pyogenes* M types 6, 12, 18, 19 and 57, collagenase digest of human glomerular basement membrane and various rabbit anti-SPM antisera are shown in Figure 1 a and b. It can be seen that, while two sets of precipitation lines can be detected between each type of SPM antigen and either its homologous or heterologous antiserum, precipitation was not observed with GBM antigen (CD).

ELISA

Circulating anti-SPM antibodies were assayed in the five rabbits immunised with SPM type 6, 12, 18, 19 or 57. One pre-immune and six serial serum samples from each animal were assayed at a 1-in-20,000 dilution, in duplicate. Each serum sample was tested against each of the SPM antigens. All the serum samples from each of the five rabbits were assayed against one SPM antigen in one single experiment.

The results obtained were essentially the same for all the SPM antigens and antisera tested. Six serial samples of each type of rabbit anti-SPM antisera had higher levels of homologous anti-SPM antibodies than pre-immune sera. In addition, there were cross-reactions among the five different M types of SPM, regardless of whether they were from nephritogenic or non-nephritogenic strains. The results obtained with the anti-SPM 12 antiserum and various SPM antigens are illustrated in Figure 2.

The existence of a cross-reaction between SPM and human GBM was investigated with both GBM antigen and rabbit anti-SPM antisera, and SPM M type 12 antigen and rabbit anti-GBM antisera.

With GBM antigen, anti-GBM antibodies were measured in the pre-immune and test bleeds of all five rabbit anti-SPM antisera (M types 6, 12, 18, 19 and 57). All serum samples were assayed with a 1-in-10 dilution of serum. The results are shown in Figure 3 and suggest a cross-reaction between GBM and SPM. When compared with the pre-immune serum, the six serial sera of each rabbit showed higher levels of anti-GBM antibodies. The pattern of response was similar amongst all five animals and, moreover, it was similar to the anti-SPM antibody response of each animal.

With SPM M type 12 antigen, anti-SPM antibodies were measured in four rabbit anti-GBM antisera and three samples of normal rabbit sera. The sera were assayed at a dilution of 1 in 1,000. The results are shown in Figure 4 and illustrate that all four antihuman GBM antisera had higher anti-SPM antibody levels than the three normal rabbit sera.

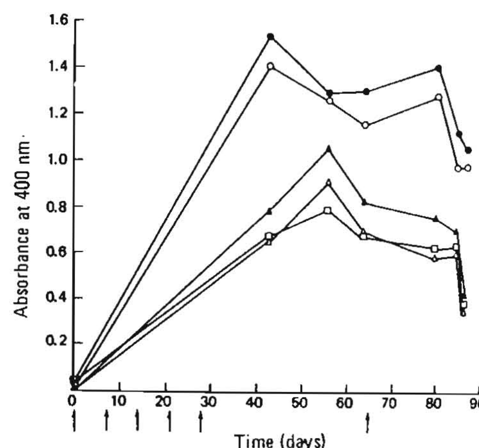

Fig. 2 Serial anti-streptococcal protoplast membrane (SPM) antibody concentrations in a rabbit immunised with SPM M type 12.
● = anti-SPM 6 antibody,
○ = anti-SPM 12 antibody,
□ = anti-SPM 18 antibody,
▲ = anti-SPM 19 antibody,
△ = anti-SPM 57 antibody,
↑ = injection of SPM M type 12.

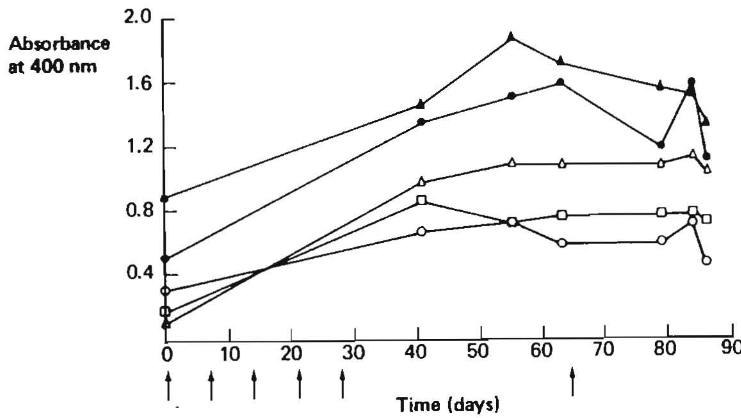

Fig. 3 Serial anti-human glomerular basement membrane antibody concentrations in five rabbits immunised with streptococcal protoplast membrane (SPM) of M type 6 (●); 12 (○), 18 (□), 19 (▲) or 57 (△). ↑ = injection of SPM.

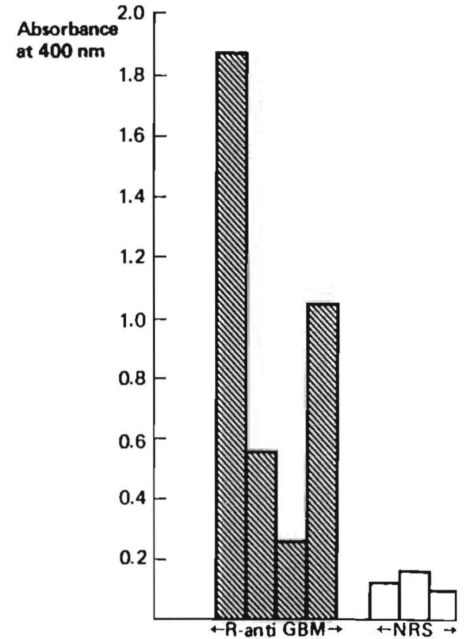

Fig. 4 Anti-streptococcal protoplast membrane M type 12 antibody concentrations in three normal rabbit sera (NRS) and in sera from four rabbits immunised with human glomerular basement membrane (R – anti GBM).

SDS-PAGE

The SDS-PAGE of all five types of SPM and a collagenase digest of human GBM (CD) is shown in Figure 5. Multiple polypeptide components, with molecular weights ranging from 78,000 to 12,300 daltons can be seen in each type of SPM and approximately 10 polypeptides in CD. One sharp band, with a molecular weight of about 60,000 daltons, appears to be common to all five types of SPM and CD.

DISCUSSION

It is well known that post-streptococcal glomerulonephritis can develop after infection with various nephritogenic strains of *Streptococcus pyogenes,* including M types 12 and 57 [13-15] The protoplast membrane of the streptococcus is among the many components which are believed to be involved in the pathogenesis of this disease.[1,3] However, there is still considerable controversy about the pathological mechanism involved, with evidence suggesting either a cross-reactive antigen between SPM of nephritogenic strains and GBM[1] or the formation of SPM antigen antibody complexes, which deposit in the glomeruli.[3]

In this report, we have investigated the existence of a cross-reaction between SPM and human GBM and the possibility that this cross-reaction might play a role in the pathogenesis of PSGN. With an ELISA technique, we have found that both nephritogenic (M types 12 and 57) and non-nephritogenic strains (M types 6, 18, and 19) of group A *Streptococcus* have protoplast membranes which possess antigens cross-reactive with each other and also with human glomerular basement membrane. In addition,

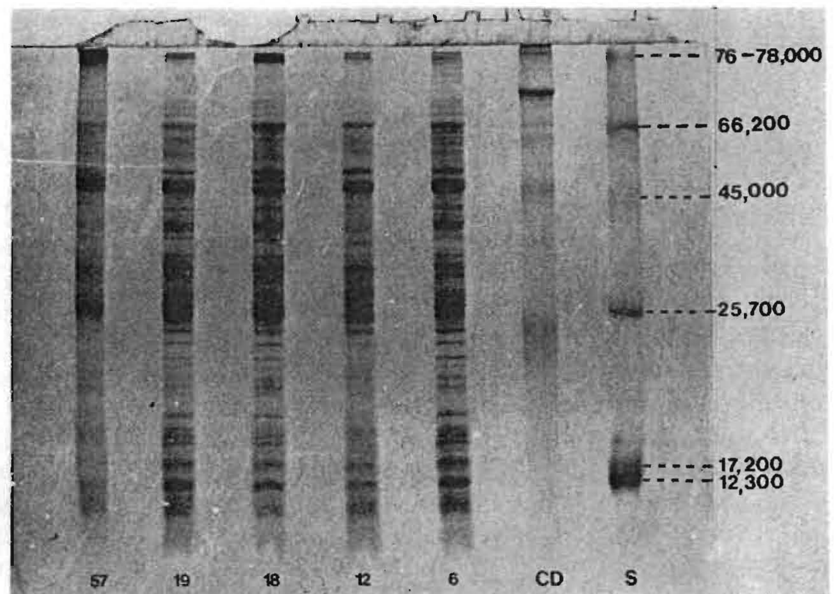

Fig. 5 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of triton X-100 extracts of streptococcal protoplast membranes (M types 6, 12, 18, 19 and 57) and a collagenase digest of human glomerular basement membrane (CD). The corresponding molecular weight of each standard protein marker is shown.

by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, one polypeptide band with a molecular weight of approximately 60,000 daltons can be seen to be common to all these SPM and to collagenase digest of GBM. However, while the cross-reaction of all five types of SPM was also detected by countercurrent immunoelectrophoresis, the cross-reaction of SPM and GBM was not observed. This discrepancy may possibly be due to the poor precipitating properties of this cross-reactive antibody.

The countercurrent immunoelectrophoresis studies, carried out with triton X-100 extracts of SPM and anti-SPM antisera, showed two sets of precipitation lines. Subsequent studies have revealed that the precipitin line nearest the SPM antigen well (Fig. 1) was an artefact. This appeared to be due to the nature of the SPM extract itself and it could not be removed by dialysis of the extract.[16] Because of this non-specific precipitation, countercurrent immunoelectrophoresis would appear to be unsuitable for the study of SPM antigens, as the artefact may mask some specific precipitation taking place in the short distance between the antigen and antibody wells.

In addition, the same results as those of countercurrent immunoelectrophoresis studies, were also obtained with other immunochemical techniques performed, i.e. immunoelectrophoresis, rocket immunoelectrophoresis and crossed immunoelectrophoresis (unpublished data).

Although our results support the existence of cross-reactive antigens between SPM and human GBM, and, thus, confirm the results of other investigators,[1] the evidence suggests that cross-reactivity may not be the sole mechanism in the pathogenesis of PSGN, as both nephritogenic and non-nephritogenic strains of *Streptococcus* have been shown to possess such cross-reactive antigens. It is certain, therefore, that further investigation is required before the role of SPM in the pathogenesis of PSGN will be clearly elucidated.

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