THE ROLE OF GLYCOPROTEIN CARBOHYDRATE IN THE IMMUNOLOGICAL REACTIVITY OF ANTISTREPTOCOCCAL CELL-MEMBRANE AND ANTIGLOMERULAR BASEMENT-MEMBRANE ANTISERA

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THE PRODUCTION of antibodies to streptococcal cell membranes (SCM) that cross-react with some human glomerular basement membranes (GBM) seems to play a major role in the induction of post-streptococcal glomerulonephritis (Markowitz and Lange, 1964; Blue and Lange, 1975). Antibody to SCM of group-A streptococci of type 12 cross-reacts with human GBM in a variety of in-vitro immunological systems (Markowitz and Lange, 1964; Holm, 1967; Blue and Lange, 1975, 1976*a* and *b*; Wheeler *et al.*, 1975). Both the streptococcal and the glomerular membrane antigens are glycoproteins (Spiro, 1967 *a* and *b*; Lange, 1969; Lange and Markowitz, 1969; Kefalides, 1972; Marquardt, Wilson and Dixon, 1973; Misra, 1973), but the cross-reactive phenomenon seems to depend upon their respective protein epitopes, with the carbohydrate moieties partially masking or preventing the attachment of antibodies (Blue and Lange, 1975). The concept of cross-reacting antibodies has received further support from the observation that anti-type-12-SCM antisera induce haemorrhagic necrosis in the skin of guinea-pigs (Lange, 1973), a phenomenon mimicking an Arthus reaction (Rapaport, Markowitz and McCluskey, 1969). A previous report (Blue and Lange, 1976*a*) left unsettled the role that the carbohydrate might play in the immunological reaction between anti-SCM and GBM or anti-GBM and SCM; this could not be investigated because of the lack of appropriate specific antisera. In this report, results are given of immunological analysis of antisera against "carbohydrate-rich" antigens from group-A, type-12 SCM and human GBM, and comparisons are made with the results obtained with anti-"protein" sera, as previously described.

MATERIALS AND METHODS

Preparation of antigens and antisera

Anti-"protein" antisera. The method of preparation of antigens for the production of anti-"protein" antisera have been described previously (Lange, 1969, 1973). In reality, these antigens contained normal amounts of glycoprotein carbohydrate, but were termed anti-"protein" because it had been observed that they reacted almost exclusively and specifically with protein epitopes (Blue and Lange, 1975, 1976*a*, 1976*b*).

Anti-"carbohydrate-rich" antisera. Antisera (PNG-SCM) were raised against a soluble fraction from SCM. Briefly, the membranes were digested with Pronase (CalBioChem, San Diego, Calif., USA) at an enzyme:substrate ratio of 1:50 (dry weight) at 37°C for 18 h. To prepare corresponding antisera against GBM (PNG-GBM), a wet weight of adult human glomerular basement membrane was digested with Pronase at a 1:50 enzyme-substrate ratio at 37°C for 18 h. After removal of the Pronase-insoluble material, the supernate was heated at 60°C for 30 min. to inactivate the enzyme and then treated with an equal volume of trifluoro-trichloroethane (Genetron, Allied Chemical Corp., Morristown, NJ, USA) as described previously (Markowitz and Lange, 1964). The aqueous phase was then dialysed against water in

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caseing with a cut-off of 3500 daltons. After lyophilisation of the non-dialysable material it was dissolved in phosphate-buffered saline (PBS), pH 7.4, and run over a Sephadex G-50 column equilibrated with PBS and eluted with PBS. Peak no. 3 (c. 5000 daltons) from the column was recovered and used to immunise rabbits. Analyses (Markowitz and Lange, 1964, Lange and Markowitz, 1969) showed these antigens to be 35% carbohydrate and 65% protein. Each rabbit received three injections, each of 5 mg of antigen in Freund's complete adjuvant, at 10-day intervals, the first intradermally and the subsequent ones intramuscularly, and the animals were bled 7 days after the last injection.

Isolation of GBM was by the methods previously reported (Markowitz and Lange, 1964; Blue and Lange, 1975).

Kidney sections

Adult and neonatal kidneys were obtained at necropsy. Cortex samples were mounted on buttons, frozen and stored at -80°F (-62°C). Sections $2\text{ }\mu\text{m}$ in thickness were cut on an IEC Harris cryostat, and were fixed on microscope slides with acetone.

Fluorescent-antibody tests

An indirect method was used, with undiluted rabbit antisera and fluorescein-conjugated sheep anti-rabbit γ globulin (Blue and Lange, 1975). The procedure for treatment of kidney sections and GBM with carbohydratase (CHOase) has been described previously (Quish and Lange, 1973; Blue and Lange 1975).

Haemorrhagic necrosis in guinea-pigs

The testing of antisera for the production of haemorrhagic necrosis in the skin of guinea-pigs has been described previously (Lange, 1973).

RESULTS

The results of comparative indirect fluorescent-antibody tests and haemorrhagic-necrosis tests in guinea-pigs is presented in the table. The four anti-"protein" antisera (nos. 23, 28, 8 and 20) reacted with adult kidney mainly as if the protein epitopes with which they reacted were partially masked by carbohydrate units. The unabsorbed antisera gave brighter GBM fluorescent staining on sections that had been treated with CHOase than on untreated sections. Absorption of the sera with native GBM slightly reduced their staining ability, while absorption with CHOase-treated GBM markedly reduced staining activity, particularly on CHOase-treated sections. All four antisera produced haemorrhagic necrosis in guinea-pigs, ranging from weakly positive (serum no. 23) to strong necrosis (serum no. 28).

The anti-"carbohydrate-rich" sera, on the other hand, reacted in a contrary fashion. Although not all of the antisera gave positive reactions in the fluorescent antibody tests with adult kidney, those that did (nos. 1103, 1124, 1110, and 1120) showed greatly reduced activity on sections that had been treated with CHOase. Also, absorption with untreated GBM removed fluorescent reactivity rather more efficiently than did absorption with CHOase-treated GBM. None of the antisera produced against "carbohydrate-rich" antigens caused haemorrhagic necrosis in guinea-pigs.

The table also shows that anti-"protein" sera reacted strongly with neonatal kidney, but that CHOase treatment of the kidney had no effect on the reactivity of the sera. Anti-"carbohydrate-rich" sera, on the other hand, showed little reaction with neonatal kidney. The kidney of the neonate, as previously shown, reacts as if the protein epitopes are "unmasked", presumably because the GBM of the neonatal kidney contain less carbohydrate than the GBM of the adult kidney (Blue and Lange, 1976a and b).

TABLE

Immunological analysis of the glycoproteins from streptococcal cell membranes (SCM) and from glomerular basement membranes (GBM): (1) immunofluorescent staining of GBM in normal adult and neonatal kidney by antisera against the glycoproteins (anti-"protein" sera), and against Pronase-treated products obtained from them (anti-"carbohydrate-rich" sera); (2) production of haemorrhagic necrosis in guinea-pigs by these sera

no.	Antiserum against	Immunofluorescence* of the stated antiserum with										Haemorrhagic necrosis* in guinea pigs 120 h after injection of antiserum
		adult human kidney				neonatal kidney						
		Serum unabsorbed		Serum absorbed with GBM		Serum absorbed with CHOase-treated GBM		Serum unabsorbed		CHOase treated kidney		
		Untreated kidney	CHOase† treated kidney	Untreated kidney	CHOase- treated kidney	Untreated kidney	CHOase treated kidney	Untreated kidney	CHOase treated kidney	Untreated kidney	CHOase treated kidney	
A. Anti-"protein" sera												
23	SCM	1+	2-3+	±	1+	0	1+	0	2-3+	2-3+	1+	
28	SLS-SCM II†	1+	2-3+	1+	2+	±	2+	±	2+	2+	4+	
8	Whole glomeruli	4+	4+	2+	2-3+	0	NT	1+	4+	4+	3+	
20	PGT-HuGl‡	3+	4+	NT	NT	NT	NT	NT	3+	3+	3+	
B. Anti-"carbohydrate-rich" sera												
1130	PNG-SCM	3+	1+	1+	0	±	0	±	±	±	0	
1104	PNG-SCM	0	±	±	±	1-2+	1-2+	0	0	0	0	
1124	PNG-SCM	1-2+	0	0	0	0	0	0	±	0	0	
1126	PNG-SCM	0	0	0	0	0	0	0	0	0	0	
1110	PNG-GBM	1-2+	1+	1+	±	±	0	0	0	0	0	
1117	PNG-GBM	1+	0	+	+	1+	1+	±	±	0	0	
1118	PNG-GBM	1+	0	±	±	1+	1+	±	±	0	0	
1119	PNG-GBM	1-2+	±	0	0	1+	1+	1+	0	0	0	
1120-21	PNG-GBM	2-3+	0	1-2+	±	1+	1+	0	1+	0	0	
C. Normal rabbit serum												
0		0	0	0	0	0	0	0	0	0	0	

NT = not tested.

* On arbitrary scales 0 to 4+.

† Treated with carbohydrtase.

‡ Second fixation from DEAE-cellulose chromatography of: SLS-SCM = sodium-lauryl-sulphate-solubilised SCM; PGT-HuGl = trypsin-digested human glomeruli (see Markowitz and Lange, 1964; Lange, 1969, 1973).

DISCUSSION

Although the immunological cross-reaction between human GBM and type-12 SCM has been observed in several laboratories (Markowitz and Lange, 1964; Holm, 1967; Blue and Lange, 1975; Wheeler *et al.*, 1975), its significance in post-streptococcal glomerulonephritis has not been generally accepted. Previous discussions of the role of the sialoprotein, and the non-linear spatial pattern of the GBM fluorescence seen as a result of the reactivities of anti-GBM or anti-SCM sera (Chiu and Drummond, 1972; Markowitz *et al.*, 1971), left open the question of the role of carbohydrate epitopes. We have attempted to correlate further the immunological reactivity of anti-SCM and anti-GBM antisera with some form of biological activity. In this process, we observed that two separate elements—the protein or the carbohydrate portions of the respective glycoprotein membranes—were concerned in the cross-reaction. Although SCM anti-“protein” sera cross-reacted with adult human GBM, this reaction was partially masked by the carbohydrate units (Blue and Lange, 1975). When this masking effect was removed by treatment of human GBM with carbohydrate-cleaving enzymes, the antisera reacted to their full potential. Carbohydrate masking of protein antigens is not unique to GBM. It has been reported in both virus-infected and transformed cell systems (Currie and Bagshawe, 1968; Collins and Black, 1974). Anti-“carbohydrate-rich” antisera, which also show reactivity with human GBM, on the other hand, become inactive when the carbohydrate is removed.

Though both anti-protein and anti-carbohydrate antibodies have been considered important in the pathogenesis of post-streptococcal glomerulonephritis it is postulated that only the anti-protein antibodies play a significant role. In view of the fact that the antisera employed in these studies were raised in rabbits, it is interesting to observe that the immune response to the native immunogens is primarily, if not exclusively, to the protein (Quish and Lange, 1973; Blue and Lange, 1975). The conclusion was drawn that the carbohydrate that masks the protein epitopes in the various immunological assays may help to direct the immune response to the protein epitopes attached to the carbohydrate. This is evident, because these antibodies can be found only after CHO is removed. A response to the carbohydrate seems to be absent in any antiserum produced to native GBM or SCM immunogens. The results we obtained with the purified carbohydrate immunogens reaffirms (Markowitz and Lange, 1964; Lange, 1969; Lange and Markowitz, 1969) the role of the oligosaccharides in the cross reactions between these two diverse materials; thus the role of the carbohydrate cannot be completely dismissed, as previously stressed (Quish and Lange, 1973; Blue and Lange, 1975).

It seems reasonable to expect that the epitope to which the host mounts his immune response is the one of significance in the pathogenesis of acute post-streptococcal glomerulonephritis. If man responds to the native immunogens as does the rabbit, this would be by the production of antibodies to the “protein” rather than to the “carbohydrate”. Further, in-vivo biological activity, as manifested by haemorrhagic necrosis in the guinea-pig, could be detected in anti-“protein” but not in anti-“carbohydrate” sera. We therefore believe that the immune response to protein epitopes of the SCM, and the resultant immunological insult to the kidney, is of importance in the aetiology of post-streptococcal glomerulonephritis.

SUMMARY

Rabbit antisera to “carbohydrate-rich” antigens prepared from group-A, type-12 streptococcal cell-membrane and human glomerular basement-membrane were found to react by an indirect fluorescent-antibody test with the glomerular basement membrane of adult human kidney. This activity was absent or diminished in neonatal tissue. Removal of the carbohydrate epitopes from the adult tissue by means of carbohydrases removed the immunological activity. Antisera against the “carbohydrate-rich” antigens showed immunological reactivities distinctly different from those against the parent native immunogens—streptococcal cell membrane or glomerular basement membrane—which proved to be directed towards the protein epitopes.

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