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Leucocyte migration inhibition with human heart valve glycoproteins and group A streptococcal ribonucleic acid proteins in rheumatic heart disease and post-streptococcal glomerulonephritis

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SUMMARY

Leucocyte migration inhibition (LMI) tests have been performed with leucocytes obtained from patients with acute rheumatic carditis, established rheumatic valvular disease with and without rheumatic reactivation, post-streptococcal glomerulonephritis and healthy volunteers, using human heart valve glycoprotein (HVGP) antigens and ribonucleic acid protein antigens (P-RNA) from Group A streptococci, types 5, 6 and 12 and a Group C streptococcus. Significantly increased LMI was observed with HVGP and P-RNA antigens of Group A streptococci in patients with clinical evidence of rheumatic valvular damage. Healthy volunteers and patients with post-streptococcal glomerulonephritis did not show any increase in LMI to these antigens. This suggests that cell-mediated immune mechanisms may be involved in the production of tissue lesions in rheumatic fever.

INTRODUCTION

An autoimmune mechanism has been implicated in the pathogenesis of rheumatic carditis. The evidence comes from demonstration of autoantibodies to heart tissue in rheumatic patients (Kaplan, Meyeserian & Kushner, 1961; Hess *et al.*, 1964; Zitran & Rosensky, 1966) and also from the demonstration of cross reactions between autoantibodies to heart and streptococcal antigens (Kaplan *et al.*, 1961; Zabriskie & Friemer, 1966; Goldstein, Halpern & Robert, 1967). Diffuse deposits of gammaglobulin have been demonstrated in the heart tissue of patients with rheumatic carditis (Kaplan *et al.*, 1964). The role of cell-mediated immune response has not been investigated a great deal, although there is some evidence in that direction. The histological appearance of the lesions of rheumatic fever bears a close resemblance to delayed hypersensitivity reaction in guinea-pigs (Long, 1954). In experimental animals, the production of cardiac lesions by repeated streptococcal infection is always associated with marked delayed cutaneous hypersensitivity (Glaser *et al.*, 1956). McLaughlin *et al.* (1972) investigated the *in vitro* response of peripheral blood leucocytes to heart and type 4 streptococcal antigens in twenty-one children with rheumatic carditis and did not find evidence to support the idea that rheumatic carditis is the result of delayed hypersensitivity to these antigens. Read, Fishetti & Zabriskie (1974) also studied leucocyte migration inhibition (LMI) in patients with streptococcal infections and rheumatic fever. They demonstrated heightened cellular reactivity to streptococcal membrane antigens in rheumatic individuals. We have investigated the role of cell-mediated immune mechanisms in the pathogenesis of rheumatic valvular damage employing LMI as a parameter of cell-mediated immunity, using the human heart

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valve glycoproteins (HVGP) and streptococcal ribonucleic acid protein antigens (P-RNA) for stimulating leucocytes from different categories of patients with rheumatic valvular disease.

MATERIALS AND METHODS

Eight patients with acute rheumatic carditis, sixteen patients with established rheumatic valvular disease with rheumatic reactivation, fifteen patients with established rheumatic valvular disease without reactivation and nine patients with post-streptococcal glomerulonephritis were studied. Nine healthy adult volunteers were also studied as controls. The patients belonged to either sex and their ages ranged between 7 and 53 years. These patients were admitted and diagnosed at JIPMER Hospital, Pondicherry, India. The criteria for the selection of cases with carditis were fever, presence of a murmur, raised erythrocyte sedimentation rate, high ALSO titre (more than 250 Todd units) and electrocardiographic changes.

Structural glycoprotein fraction from human heart valves was prepared from heart valves removed at autopsy according to the procedure described by Goldstein *et al.* (1967). Human heart valves were removed at autopsy from cases known to have died of causes other than rheumatic heart disease within a few hours after death. The heart valves were homogenized in citric acid, Tris and calcium chloride buffer pH 7·5. After homogenization seven extractions of 24 hr each at 4°C were carried out in the same buffer. Centrifugation at 500 *g* for 15 min was done and the insoluble residue obtained as the sediment was heated three times to 90°C in 3·8% trichloroacetic acid, cooled and centrifuged. The sediment was extracted six times with 8·0 M Urea for 24 hr each at 4°C and centrifuged. The supernate urea extract containing structural glycoproteins of human heart valves was dialysed against 0·9% saline before use.

P-RNA fraction from group A streptococci types 5, 6 and 12 and group C streptococcus were prepared as described by Schalla & Johnson (1975). Trypsin treatment of streptococci was done according to the method of Fox (1961), and the phenol extraction method of Kirby (1956) was used for extracting P-RNA fractions. Group A streptococci types 5, 6 and 12 were obtained from Dr K.B. Sharma, Department of Microbiology, Lady Hardinge Medical College, New Delhi and Dr J. Rotta, Director, International Reference Centre for Streptococci, Prague. Group C streptococcus was obtained from Dr Maxted, Central Public Health Laboratory, Colindale, London, UK. The different strains of streptococci were grown in 3 litres of Todd–Hewitt broth at 37°C for 12 hr. Growth was collected by centrifugation at 800 *g*. The cells were washed three times with 0·02 M phosphate buffer containing 0·01 M magnesium chloride, pH 7·4 (PMB). The cells were suspended in 200 ml of 0·01 M sodium bicarbonate containing 5 mg of crystalline trypsin and incubated for 30 min at 37°C to remove the M protein from the surface of streptococci (Fox, 1961). After centrifugation at 800 *g* the cells were washed three times with PMB and suspended in four times their volume of PMB. Ten milligrams of lysozyme hydrochloride was added and the mixture incubated at 37°C for 1 hr and 2 mg/ml of deoxyribonuclease was added after incubation. The cells were ground for 1 hr with 10 mg of sterile powdered pyrex glass. An equal volume of 1% (w/v) sodium dodecyl sulphate and 0·7 M ammonium chloride dissolved in 0·02 M PMB were added to the disrupted slurry with gentle agitation. The cell debris and glass powder were removed by centrifugation. To the supernatant an equal volume of 90% (w/v) phenol was added and stirred for 1 hr. The mixture was centrifuged at 600 *g* for 15 min. The upper aqueous layer was aspirated leaving the phenol layer and an insoluble protein layer over it. Two volumes of absolute alcohol chilled at −20°C were added to the aqueous layer. The precipitate was collected by centrifugation. After evaporating any residual alcohol it was dissolved in 0·16 M saline.

The protein content of various preparations was determined by the method of Lowry *et al.* (1951). Standard curves were made with bovine serum albumin (Fraction V Powder, Sigma Chemical Company, St Louis, Missouri). The RNA content of different preparations was estimated by pentose analysis as described by Schneider (1957).

Leucocyte migration inhibition tests were performed according to the method described by David *et al.* (1964) and as modified by Agarwal & Sundararaj (1976). Ten millilitres of blood from patients was collected in a sterile, siliconized tube containing 250 units of heparin. It was centrifuged at 3000 r.p.m. for 30 min in a swing-out centrifuge. The buffy coat was removed with a Pasteur pipette and placed in another tube. It was washed twice with Hanks' balanced salt solution (HBSS) containing 5 u/ml of heparin. The cells were suspended in about 5 ml of 0·83% ammonium chloride and allowed to stand for 5 to 10 min. After the lysis of erythrocytes, the leucocytes were removed by centrifugation at 1500 r.p.m. for 2 min and washed twice with HBSS. The cells were then suspended in Eagle's minimum essential medium supplemented with 10% inactivated bovine serum at a concentration of $2–3 \times 10^7$ cells/ml. HBSS and Eagle's minimal essential medium were prepared according to Lennette & Schmidt (1974). The cells were drawn into microcapillaries (1–2 mm internal diameter and 120 mm long), one end of which was heat-sealed and centrifuged in a swing-out centrifuge. Capillaries were cut at the cell–fluid interface. These were fixed with silicone grease to the inner rim of plastic ring chambers. The chambers were filled with minimum essential medium containing 10% inactivated bovine serum, penicillin (100 u/ml) and streptomycin (100 μg/ml). The various streptococcal antigens and heart valve glycoprotein antigens were added in different chambers. The dilution of antigen used was the one which by itself did not inhibit leucocyte migration. The chambers were incubated at 37°C for 18 hr to allow leucocyte migration to proceed. The area of migration was traced by using camera lucida and measured. The average of the area of migration of five capillaries was taken and the migration index calculated using the formula:

$$\text{Migration index} = \frac{\text{Area of migration with antigen}}{\text{Area of migration without antigen}} \times 100.$$

The results of LMI in different categories of patients of rheumatic valvular disease and healthy individuals were analysed

statistically and the mean, standard error of mean, t and P values were determined in each group as compared with the figures in healthy volunteers. ASLO determinations were made according to the procedure described in WHO manual of reference procedures in streptococcal bacteriology and serology—SEA/HLM/124 (1974).

RESULTS

The values of ribonucleic acid, protein and RNA/protein ratio in the different streptococcal antigens and the optimum concentrations which were non-inhibitory to leucocytes in the absence of any immune reactivity are shown in Table 1. Concentrations of 6·25 μg/ml, 3·0 μg/ml, 12·5 μg/ml and 12·5 μg/ml of P-RNA of Group A streptococci 5, 6, 12 and Group C streptococcus, respectively were employed. A dilution of 1:8 HVGP antigen was used.

A highly significant increase in LMI to HVGP was present in rheumatic patients with clinical evidence

TABLE 1. The value of RNA and protein, RNA/protein ratio and concentrations of the different streptococcal antigens used in LMI tests

Sl. no.	Streptococcus group	RNA (μg/ml)	Protein (μg/ml)	RNA:protein ratio	Concentration used in LMI tests (μg/ml)
1	Group A, type 5	300	1225	20:80	6·25
2	Group A, type 6	600	990	39:61	3·0
3	Group A, type 12	210	9892	20:80	12·5
4	Group C	180	1160	13:87	12·5

TABLE 2. Leucocyte migration inhibition* with human heart valve glycoprotein antigen in healthy volunteers, in patients with acute rheumatic carditis, rheumatic valvular disease with and without reactivation and post-streptococcal glomerulonephritis

Sl. no.	Healthy volunteers	Acute rheumatic carditis	RHD with reactivation	RHD without reactivation	Post-streptococcal glomerulonephritis
1	13·0	40·1	36·4	26·1	−14·2
2	5·9	11·5	9·6	−3·2	−16·1
3	−11·6	20·0	1·9	15·0	−11·0
4	−13·3	34·8	−2·3	−6·0	12·2
5	−8·3	27·8	25·9	3·1	−1·1
6	18·3	43·7	20·2	47·5	0·1
7	4·2	10·0	26·2	6·6	−2·2
8	0·5	—	1·5	68·1	−6·5
9	1·1	—	2·7	12·3	—
10	—	—	4·2	15·3	—
11	—	—	26·6	23·8	—
12	—	—	2·3	13·9	—
13	—	—	25·3	10·0	—
14	—	—	20·6	15·1	—
15	—	—	37·6	22·3	—
16	—	—	—	—	—
Mean	2·74	23·50	16·09	18·60	3·50
s.e.m.	1·43	5·57	3·33	4·88	33·55
t	—	3·75	3·98	3·48	0·21
P	—	< 0·01	< 0·01	< 0·01	> 0·5

* Results are expressed as a percentage of migration inhibition.

TABLE 3. Leucocyte migration inhibition tests against P-RNA antigen of group A type 5 streptococcus in healthy volunteers, in patients with acute rheumatic carditis, rheumatic valvular disease with and without reactivation and post-streptococcal glomerulonephritis

Sl. no.	Healthy volunteers	Acute rheumatic carditis	RHD with reactivation	RHD without reactivation	Post-streptococcal glomerulonephritis
1	9·3	42·5	7·1	4·2	20·6
2	−6·1	4·7	15·7	19·6	18·8
3	16·6	2·5	3·0	6·8	6·5
4	−2·6	0·7	16·8	12·3	−6·3
5	−0·3	31·2	−6·4	12·1	−2·3
6	4·9	25·4	9	19·9	−0·2
7	5·8	11·9	−10·4	−5·6	−6·1
8	−9·7	12·7	9·2	61·1	10·7
9	−9·4	—	1·1	−2·6	—
10	—	—	14·1	10·6	—
11	—	—	18·6	−7·3	—
12	—	—	27·5	9·9	—
13	—	—	2·5	−4·7	—
14	—	—	11·6	−9·8	—
15	—	—	7·4	19·2	—
16	—	—	6·2	—	—
Mean	4·6	16·45	9·36	11·26	7·07
s.e.m.	1·93	5·33	1·90	3·64	3·07
t	—	2·30	1·95	1·97	1·18
P	—	< 0·01	< 0·02	< 0·1	> 0·1

Results are expressed as a percentage of migration inhibition.

TABLE 4. Leucocyte migration inhibition tests against P-RNA antigen from group A type 6 streptococcus in healthy volunteers, in patients with acute rheumatic carditis, rheumatic valvular disease with and without reactivation and post-streptococcal glomerulonephritis

Sl. no.	Healthy volunteers	Acute rheumatic carditis	RHD with reactivation	RHD without reactivation	Post-streptococcal glomerulonephritis
1	1·9	14·8	11·0	−0·3	30·9
2	−1·6	−3·4	4·0	20·3	40·1
3	−8·2	16·3	−12·1	−5·0	11·6
4	−2·7	−9·1	16·0	−4·0	10·8
5	−2·6	25·1	−8·0	4·9	6·8
6	−2·1	−12·7	15·3	9·8	−0·1
7	1·5	−7·1	15·1	−2·5	19·9
8	−6·2	11·5	17·0	21·2	12·2
9	−2·9	—	14·9	10·7	—
10	—	—	30·1	8·6	—
11	—	—	21·3	8·6	—
12	—	—	27·7	4·5	—
13	—	—	1·3	0·6	—
14	—	—	22·9	0·6	—
15	—	—	1·6	2·1	—
16	—	—	8·6	—	—
Mean	0·38	8·40	16·25	6·50	16·50
s.e.m.	0·02	3·47	2·60	1·85	4·6
t	—	2·28	6·10	3·31	3·48
P	—	< 0·05	< 0·01	< 0·01	< 0·01

Results are expressed as a percentage of migration inhibition.

of valvular damage, i.e. patients with acute rheumatic carditis ($P<0{\cdot}01$), established rheumatic valvular damage with reactivation ($P<0{\cdot}01$), and without reactivation ($P<0{\cdot}01$). On the other hand, patients with post-streptococcal glomerulonephritis did not show increased LMI with HVGP ($P>0{\cdot}5$). These results are shown in Table 2. The results obtained with PRNA of Group A streptococcus type 5 are shown in Table 3. In all categories of rheumatic patients there was significantly increased LMI. The P values were as follows: patients with acute rheumatic carditis, $P<0{\cdot}1$; patients with established rheumatic valvular disease with reactivation, $P<0{\cdot}02$; patients with established rheumatic valvular disease without reactivation, $P<0{\cdot}1$. There was no significant increase in LMI in patients with glomerulonephritis ($P>0{\cdot}1$). Table 4 shows that a significant increase in LMI was obtained with PRNA of type 6 streptococcus both in patients with rheumatic heart disease and glomerulonephritis. The P values for different categories were as follows: acute rheumatic carditis, $P<0{\cdot}05$; established rheumatic valvular disease with reactivation, $P<0{\cdot}01$; rheumatic heart disease with reactivation, $P<0{\cdot}01$; post-streptococcal glomerulonephritis, $P<0{\cdot}01$.

The results with P-RNA of Group A type 12 streptococcus are shown in Table 5. Rheumatic individuals of all categories showed increased LMI, while patients with glomerulonephritis did not. The P values were: $<0{\cdot}02$ in acute rheumatic carditis; $<0{\cdot}1$ in rheumatic heart disease; $<0{\cdot}05$ in rheumatic heart disease without reactivation and $>0{\cdot}1$ in glomerulonephritis. In contrast with all other antigens, the PRNA antigen of group C streptococcus did not induce a significant degree of migration inhibition in any of the patient groups studied ($P>0{\cdot}1$ in all categories) as shown in Table 6.

DISCUSSION

The increased LMI of leucocytes from patients with rheumatic valvular disease, when stimulated with HVGP, provides evidence for the role of cell-mediated immune mechanism in the pathogenesis of

TABLE 5. Leucocyte migration inhibition tests against P-RNA antigen from group A type 12 streptococcus in healthy volunteers, in patients with acute rheumatic carditis, rheumatic valvular disease with and without reactivation posi-streptococcal glomerulonephritis

Sl. no.	Healthy volunteers	Acute rheumatic carditis	RHD with reactivation	RHD without reactivation	Post-streptococcal glomerulonephritis
1	9·5	3·8	3·6	17·5	40·3
2	2·9	11·9	23·6	−9·7	26·8
3	−8·9	−15·4	20·7	3·7	25·7
4	16·8	14·5	8·4	−6·5	2·5
5	2·9	18·0	−2·0	−2·3	0·0
6	4·3	13·8	−16·8	12·3	−1·4
7	−1·6	8·1	−3·5	−1·8	−5·9
8	−9·6	4·1	18·2	30·1	10·8
9	0·6	—	−6·8	17·6	—
10	—	—	20·1	12·5	—
11	—	—	5·8	9·2	—
12	—	—	15·9	12·7	—
13	—	—	−0·3	3·2	—
14	—	—	−1·5	−3·4	—
15	—	—	5·6	16·8	—
16	—	—	16·7	—	—
Mean	4·10	9·20	8·30	9·04	13·20
s.e.m.	1·85	1·55	2·27	2·77	5·51
t	—	3·28	1·83	2·14	1·65
P	—	$<0{\cdot}02$	$<0{\cdot}1$	$<0{\cdot}05$	$>0{\cdot}1$

Results are expressed as a percentage of migration inhibition.

TABLE 6. Leucocyte migration inhibition tests against P-RNA antigen from group C streptococcus in healthy volunteers, in patients with acute rheumatic carditis, rheumatic valvular disease with and without reactivation and post-streptococcal glomerulonephritis

Sl. no.	Healthy volunteers	Acute rheumatic carditis	RHD with reactivation	RHD without reactivation	Post-streptococcal glomerulonephritis
1	1·6	11·5	−9·8	−1·4	11·0
2	−2·6	1·8	10·5	3·8	7·3
3	−10·6	6·4	−10·3	1·8	4·4
4	n.d.	19·1	4·3	4·2	−1·9
5	−2·8	11·6	−3·7	12·2	0·4
6	−5·2	8·3	6·1	17·0	1·3
7	−24·5	21·4	−3·9	10·7	−1·7
8	−11·3	9·8	13·1	n.d.	n.d.
9	26·5	—	4·9	20·5	—
10	—	—	15·2	17·7	—
11	—	—	−6·2	−10·9	—
12	—	—	17·0	12·2	—
13	—	—	−1·3	−0·3	—
14	—	—	9·6	20·1	—
15	—	—	−10·4	1·8	—
16	—	—	15·3	—	—
Mean	3·51	11·38	6·00	8·35	3·61
s.e.m.	3·07	1·99	1·62	5·10	1·65
t	—	1·39	1·53	1·50	0·006
P	—	>0·1	>0·1	>0·1	>0·5

Results are expressed as a percentage of migration inhibition.
n.d. = Not done.

rheumatic valvular lesions. Although humoural antibodies to cardiac tissue have been described earlier, the exact role of cell-mediated immune reactions in the pathogenesis of rheumatic valvular lesions is not yet clear and the reports are conflicting. Mclaughlin *et al.* (1972) studied cell-mediated immune response to an acid extract of normal human myocardium and to a Group A type 4 streptococcal cell wall preparation, using tritiated thymidine incorporation by proliferating peripheral lymphocytes as an *in vitro* correlate of cell-mediated immunity. They could not find any significant cellular response to these antigens in rheumatic children or controls. However, studies by Read *et al.* (1974), using LMI as the *in vitro* correlate of cell-mediated immunity and using cell membrane preparations from types 5 and 6 Group A streptococci as antigens, showed an increased cellular response in rheumatic individuals. Read *et al.* (1974) did not use heart derived antigens in their investigations. In our study, an increased LMI has been demonstrated not only against HVGP, but also against P-RNA antigens of Group A streptococci. The migration inhibition with Group A streptococcal P-RNA antigens could not have been due to the toxic effect of M protein on leucocytes (Beachy & Stollerman, 1971) because M proteins had been removed from the surface of streptococci prior to P-RNA extraction (Fox, 1961).

The demonstration of an increased LMI to HVGP in patients with rheumatic valvular damage might be taken as evidence for the presence of T lymphocytes sensitized with HVGP. It was not so in patients with glomerulonephritis or healthy volunteers. Thus, there is a correlation between presence of valvular lesion and T cell sensitization of HVGP. There was also a sensitization with P-RNA from rheumatogenic types 5 and 6 streptococci. The coexistence of T cell sensitization of HVGP and streptococcal P-RNA in rheumatic patients provides an important link between streptococcal infection and heart valve damage. It seems likely, therefore, that valvular damage in rheumatic heart disease may be brought about by a type IV cell-mediated immune reaction involving T cells sensitized against HVGP. It is not possible from our studies to state definitely whether the sensitization to HVGP and streptococcal P-

RNA in the same individuals should be considered as evidence of antigenic relationship between HVGP and Group A streptococcal P-RNA or whether initial cardiac damage occurs due to streptococcal products, resulting in alteration or unmasking of chemical groupings in the valve substance to which an immune response ensues. Cell-mediated immune response to HVGP, irrespective of the way it develops, can be expected to cause valvular damage in rheumatic patients.

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