Immunology, 1967, **13**, 209.

Studies on the Antigen in β-Haemolytic Streptococci that Cross-Reacts with an Antigen in Human Myocardium

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(*Received* 31*st January* 1967)

Summary. A common antigen has been demonstrated in several serological types of Group A β-haemolytic streptococci. This antigen is cross-reactive with a cardiac and a voluntary muscle tissue antigen in man, the rabbit and the guinea-pig. The streptococcal antigen is present in smooth and matt variants of Group A streptococci, is absent from a type 24 Group A matt streptococcus, is present in Group G and absent from Groups C and D. It is distinguishable from the M type-specific antigen of Group A. The properties of the streptococcal and the cross-reacting tissue antigen have been studied. They are probably complex antigens of protein, polysaccharide and lipid.

A similar antigen (or hapten), also cross-reacting with cardiac and voluntary muscle, has been found in human saliva. The salivary and the streptococcal antigens are probably related to the blood group substances. The salivary antigen (or hapten) could be successfully coated upon living streptococci but not upon a living *Staphylococcus aureus*.

INTRODUCTION

Streptococcal infections of the throat are followed in a small proportion of individuals by lesions affecting the heart, the kidney and, more rarely, other tissues. The sequence of events relating these late lesions of the post-streptococcal state to the initial infection has proved a baffling problem, but recently accumulated evidence strongly suggests that the lesions follow immunologically mediated reactions resulting from the presence, in the infecting organisms, of antigens that cross-react with organ antigens present in the affected tissues. Since the most important of these post-streptococcal lesions is the carditis associated with rheumatic fever, the major effort has been directed towards the demonstration of cross-reacting antigens in the heart. The low incidence of rheumatic fever after streptococcal infections, however, and the earlier work of Kaplan and Meyeserian (1962), suggest that the presence of the antigen might be restricted to certain strains of streptococci. The distribution of the antigen has therefore been studied in different streptococcal groups and types, as well as in the smooth, matt and L forms of some individual types.

In view of the apparent necessity for the streptococcal infection that precedes an attack of rheumatic fever to involve the throat, and because of the established negative correlation between the salivary secretion of blood group substances and the incidence of rheumatic fever (Glynn, Glynn and Holborow, 1959), an investigation was made of human saliva

for the presence of an antigen capable of cross-reacting with a cardiac antigen in a manner comparable to the cross-reacting antigen in streptococci. The presence of such a salivary antigen was established and its close immunological relationship to the cross-reacting antigens of streptococci and myocardium demonstrated.

MATERIALS AND METHODS

Organisms

The following organisms were employed:

Haemolytic streptococci: Group A Types 1, 2, 6 (matt and glossy)
12 (glossy and L forms)
18, 19, 24, 30, 47, 51;
Group C, D and G;
Staphylococcus aureus.

The types 1, 12, 18 and 19 were obtained from the M.R.C. Rheumatism Research Unit, Canadian Red Cross Memorial Hospital, Taplow, and had been isolated from the throats of children admitted there with acute rheumatic fever. The other types of Group A, as well as single strains of Groups C and G, were obtained from the Central Public Health Laboratory, Colindale, London. The L form of type 12 was kindly provided by Mr W. R. Maxted of the Central Public Health Laboratory. The remaining organisms were obtained from the routine bacteriology laboratory of the West London Hospital, Hammersmith.

Culture media

Three media were used:

(1) Todd Hewitt, obtained in powder form from Oxoid, London, and reconstituted by dissolving 36·4 g of the powder in 1 litre of distilled water;

(2) Trypticose Soya Broth (Baltimore Biological Laboratories, Baltimore, Maryland, U.S.A.) and used as described by Kaplan (1958);

(3) Casein Digest Dialysate Supplemented (CDDS). This was used as described by Kaplan and Meyeserian (1962).

The purpose of the two last media was to avoid the presence in the medium of proteins or haptens of mammalian origin which might possibly be absorbed by the growing organisms and thus give misleading evidence of cross-reacting antigens.

Preparation of vaccines

(i) *Streptococci.* A bottle of 250 ml of the medium was inoculated from an 18-hour culture of the organism which had been subcultured several times in the corresponding medium. After 24 hours growth at 37° the organisms were separated by centrifugation, washed three times in sterile saline and then suspended in 25 ml of sterile phosphate buffer at pH 7·8. They were killed by heating for 30 minutes in a water bath at 56°. The *Streptococcus faecalis* (Group D) was killed by heating for 45 minutes at 65°.

(ii) *Staphylococcus aureus.* These vaccines were prepared in the same way but grown exclusively in CDDS and killed by heating at 60° for 1 hour.

(iii) *Vaccines of saliva-treated organisms.* Human saliva was collected in sterile Universal bottles and merthiolate 1:10,000 added as preservative. When not immediately required the saliva was stored at 4°. Two millilitres of the saliva was added to the washed organisms obtained from a 24-hour culture grown in 25 ml of the CDDS medium. The mixture was

mixed thoroughly and then incubated for 1 hour at 37° with frequent shaking. The organisms were recovered by centrifugation, washed three times in 25 ml of sterile saline, suspended in 2·5 ml of the phosphate buffer (pH 7·8) and killed as before.

Immunization of rabbits

The undiluted vaccine was given intravenously according to the following schedule: Day 1, 0·5 ml; days 8 and 9, 1 ml each day, repeated on two successive days each week for another 6–8 weeks. The animals were bled 1 week after the last injection. The separated sera were stored without preservative at −20°.

The sera were tested for group and type specific antibodies by capillary precipitin tests against acid extracts of the corresponding organisms. The sera, prior to being tested for type specific antibody, were absorbed with a thick suspension of a strain known to be lacking the M type-specific protein. Booster injections were given after a 4-week rest period. A booster course consisted of two injections per week each of 1 ml of vaccine intravenously for 2 or 3 weeks. The L forms of type 12 were employed according to the same schedule as the other organisms, but were first deposited by centrifugation from the fluid medium in which they were grown and re-suspended in 2·5 ml of sterile saline (2·5 per cent).

Immunofluorescent staining

This was done exclusively on cryostat sections of unfixed tissues. Cold alcohol fixation was inapplicable as the cross-reacting antigen in the heart is readily soluble in this fixative.

Tissues from rabbit, guinea-pig and man were studied. The human tissue was obtained either by biopsy of an auricular appendage during cardiac surgery or at autopsy within a few hours of death. The animal tissues were all obtained from freshly killed individuals. All tissues were placed in polythene containers, 'snap' frozen in a mixture of solid CO_2 and alcohol, and stored at −20°.

Sections were cut at 5 μ, dried onto slides in a draught of cold air for 1 hour, and used immediately without further fixation. Test sera were used neat or appropriately diluted. The immunofluorescent titre of most positive sera lay between 1:8 and 1:32. A single drop, about 0·05 ml, was left on the section for 30 minutes at room temperature, washed for 30 minutes in Coons's buffered saline with constant stirring. After the removal of excess fluid a drop of fluorescein-conjugated goat anti-rabbit γ-globulin was left on the section for 30 minutes, after which the preparation was again washed and mounted in buffered glycerine.

Non-specific staining given, for example, by the serum of bleeds taken before immunization was extremely troublesome at first. It was, however, almost entirely suppressed by mixing each serum before testing with an equal volume of 20 per cent bovine serum albumin in Coons's buffered saline. Results were only accepted as positive, therefore, if staining was obtained in the presence of a final concentration of 10 per cent of bovine serum albumin.

The preparations were examined with a Cooke, Troughton and Sims microscope model M 2000 fitted with a dark ground condenser. The ultraviolet light source was a Mazda lamp, 250 W type ME/D, filtered through a Chance 18A filter. A Wratten 1B filter was fitted into the eyepiece. Photomicrography was on Ektachrome high speed colour film (ASA 160).

Absorption

Sera were absorbed by addition to each of an equal volume of absorbent, incubated at 37° for 1 hour with frequent shaking, followed by overnight storage at 4°. The sera were then recovered by centrifugation. Organisms were used as a thick suspension, organ extracts as a crude emulsion made with an equal volume of buffered saline. Acid extracts of streptococci were prepared according to Lancefield (1938). Extracts in buffered saline were similarly prepared by boiling the organisms in Coons's buffered saline instead of N/5 HCl.

Complement fixation

This was done according to Donnelly (1951), using M.R.C. pattern Perspex plates (Asherson and Dumonde, 1962). The following antigens were used:

(1) *Cardiac*, three preparations were made: (a) a 1 per cent suspension of fresh human heart (Kaplan, 1958); (b) a saline extract of human heart. A cold extract in veronal buffer, 50 g of heart to 50 ml of buffer, was prepared after the tissue had been minced and thoroughly washed in isotonic saline. The homogenate, after standing overnight at 4°, was boiled for 15 minutes and then filtered. The filtrate was used as antigen; (c) an alcoholic extract of fresh human heart, as used for routine Wassermann reactions. It was kindly supplied by Mr H. G. Blunt of the West London Hospital and used at a dilution of 1:60 without addition of cholesterol. Similar preparations were also made from hearts of rabbits and guinea-pigs.

(2) *Voluntary muscle*: suspensions and saline extracts from human, rabbit and guinea-pig voluntary muscle were prepared in the same way as the corresponding cardiac preparations.

(3) *Human saliva*

The complement used was either the preserved material as supplied by Burroughs Wellcome Ltd, or a pool of serum from freshly killed guinea-pigs. The indicator system was a 5 per cent suspension of sheep red cells sensitized with 5 MHD of Difco rabbit amboceptor. All sera were inactivated in a water bath at 56° for 30 minutes.

Coated sheep cell agglutination

The tanned cell method of Boyden as modified by Sewell, Cooke and Cox (1963) was used initially, but subsequent work showed that adequate sensitization could be as readily obtained without preliminary tanning. This step was, therefore, omitted. The antigens employed were prepared as for the complement fixation test, and included saline extracts of human cardiac and voluntary muscle, alcoholic extract of human cardiac muscle and human saliva. All test sera were absorbed for 30 minutes with packed washed sheep red cells to remove heterophile antibodies.

Sensitization of the cells was obtained by mixing equal volumes of a 2 per cent suspension of washed sheep cells and a solution of the coating antigen. After incubation in a water bath at 37° for 30 minutes the cells were spun down, washed three times in horse serum saline solution (HSS) (2 per cent inactivated horse serum in buffered saline, pH 7·2) and used as a 1 per cent suspension in the same medium. Cells were used on the same day as sensitized. Sera were serially diluted two-fold in HSS and added to an equal volume of the cell suspension. Agglutination was read after 2 hours at room temperature followed by overnight incubation at 4°.

Three types of control were used in every test: (1) unsensitized cells; (2) sensitized cells and pre-immune sera; and (3) sensitized cells and immune sera that had been previously neutralized by various antigens. The controls were consistently negative.

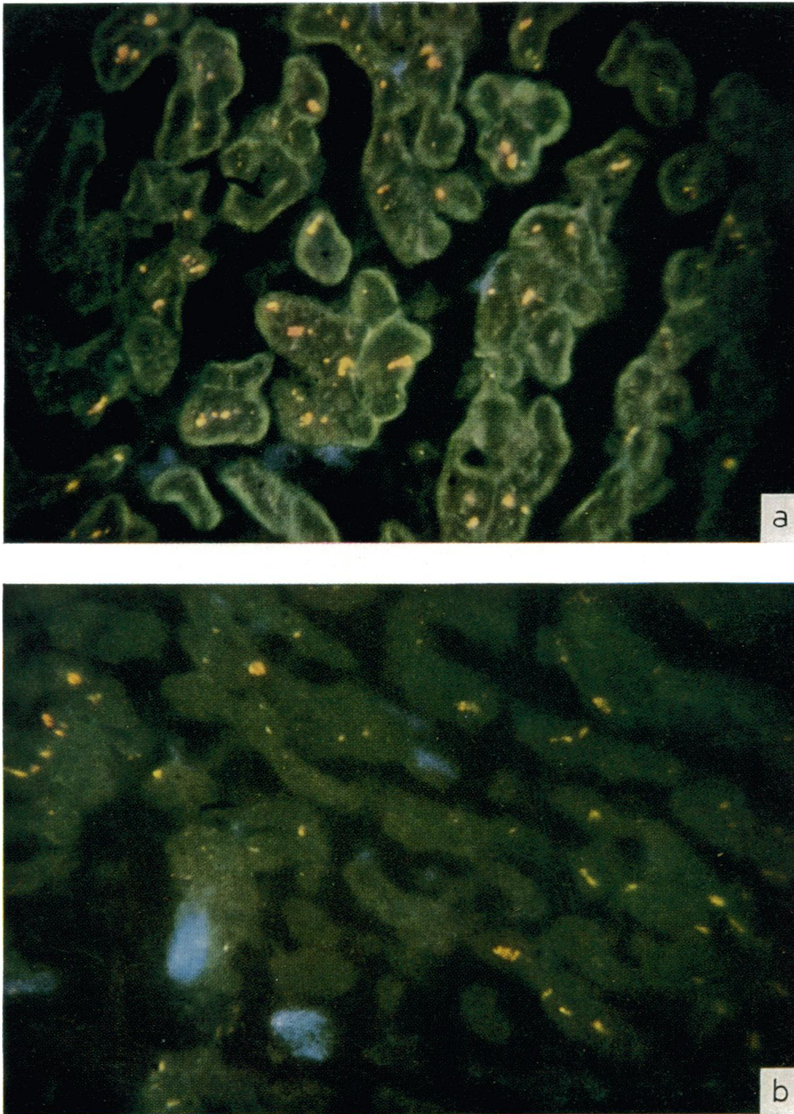

FIG. 1. Cryostat sections of unfixed human heart. Immunofluorescent staining (sandwich method) with rabbit sera and goat anti-rabbit globulin fluorescent conjugate. (a) Treated with serum from a rabbit immunized with a vaccine of Group A haemolytic streptococci. (b) Treated with normal rabbit serum. ×240.

Skin tests

Five rabbits shown to give neither immediate nor delayed skin reactions to human saliva when tested with 0·1 ml of the neat or diluted material were immunized by intradermal injection of an emulsion of equal parts of neat saliva and Freund's incomplete adjuvant, to which was added 1 mg/ml of human tubercle bacilli. The immunizing dose was 0·4 ml distributed over multiple sites. These rabbits were subsequently re-tested with saliva, extracts of various organisms and extracts of various organs. The results were read at 4, 24 and 48 hours.

RESULTS

IMMUNOFLUORESCENT STAINING

1. *Antisera to streptococci*

Antisera to ten out of the twelve varieties of Group A streptococci, and to the single example of Group G gave positive fluorescent reactions with cardiac and voluntary muscle of all three species tested; the reactions with smooth muscle, liver, spleen, kidney and lung were all negative. Negative results with all tissues, including cardiac and voluntary muscle, were given by the antisera to type 24 and type 12 (L form) of Group A, and to the single examples of Groups C and D.

Two variants of the type 6 organism were available, a matt and a glossy. Antisera to both were equally positive despite the absence of the M protein from the glossy variant. Conversely the antiserum to the type 24 was negative, despite the presence of the M antigen in this organism.

The pattern of immunofluorescent staining was similar with all the positive sera (Fig. 1a and b). It was localized to the sarcolemma and subsarcolemmal regions of the myofibres with occasionally some fluorescent stippling within the body of the fibres. The staining pattern was the same in both cardiac and voluntary muscle and in all three species studied. Staining was sometimes encountered in the smooth muscle fibres of the media of the cardiac blood vessels, but was not observed in the other organs.

The pre-immune sera gave consistently negative staining when used with an equal volume of 20 per cent bovine serum albumin.

2. *Antisera to saliva-treated organisms*

The three negative examples of streptococci, namely Group A type 24, Group C and Group D, were used to prepare saliva-treated vaccines for immunization. A *Staphylococcus aureus* was also used in the same way. The antisera to all three treated streptococci gave unequivocal positive fluorescence with cardiac and voluntary muscle; the anti-staphylococcal serum was negative. An antiserum against saliva alone (the same sample as was used to coat the organism) gave positive but weak immunofluorescent staining. This antiserum was obtained by intravenous immunization; another antiserum, kindly provided by Dr Stanley Roberts and obtained by immunization with saliva in Freund's complete adjuvant, gave much stronger staining indistinguishable from that obtained with saliva-coated streptococci.

3. *The effect of absorption and cross-absorption on immunofluorescence*

To test the specificity of the fluorescence reactions the reactive antisera were subjected to absorption with the various antigenic preparations. Each of the positively reacting

anti-streptococcal antisera was absorbed with the corresponding organisms or with their acid or buffer extracts. The results are summarized in Table 1. In every instance fluorescent staining was inhibited by such absorption. When the sera were absorbed with organisms of another type, fluorescence was again inhibited, except when the absorbing organisms were of Group C or D or of Group A type 24.

TABLE 1
RESULTS OF IMMUNOFLUORESCENCE ABSORPTION TESTS

Antiserum	Immunofluorescence of antisera absorbed with:							
	Homologous streps.	Homologous acid extract	Homologous buffer extract	Heart homogenate	Voluntary muscle homogenate	Liver homogenate	Medium of growth*	Heterologous bacteria†
Type 1 Group A	−	−	−	−	−	+	+	+
Type 2 Group A	−	−	−	−	−	+	+	+
Type 6 Group A	−	−	−	−	−	+	+	+
Type 6 glossy Group A	−	−	−	−	−	+	+	+
Type 12 glossy Group A	−	−	−	−	−	+	+	+
Type 18 Group A	−	−	−	−	−	+	+	+
Type 19 Group A	−	−	−	−	−	+	+	+
Type 30 Group A	−	−	−	−	−	+	+	+
Type 47 Group A	−	−	−	−	−	+	+	+
Type 51 Group A	−	−	−	−	−	+	+	+
Saliva-treated Group C	+	+‡	+‡	−	−	+	+	+
Saliva-treated Group D	+	+‡	+‡	−	−	+	+	+
Saliva-treated type 24 Group A	+	+‡	+‡	−	−	+	+	+
Group G	−	−	−	−	−	+	+	+

+, Fluorescence; −, no fluorescence.
* Each antiserum was absorbed by the medium on which the organisms were grown.
† These included *Staphylococcus aureus*, *E. coli* and *Streptococcus faecalis*. Antisera against saliva-treated organisms were also absorbed with *Streptococcus viridans* and *Neisseria catarrhalis* isolated from the corresponding saliva.
‡ Extract of the untreated organisms.

The activity of antisera against saliva-treated streptococci, like that against any of the positive types, was readily absorbed by any of these types but not by untreated organisms of type 24 or of Groups C or D. Absorption was readily obtained, however, by using these same organisms coated with saliva. Similarly, inhibition of fluorescent staining by any of the positive sera was achieved by the addition of fresh or boiled saliva, or by extracts of voluntary or cardiac muscle, whether of human, rabbit or guinea-pig.

To check the possibility that the cross-reacting material in saliva was derived from organisms naturally present within it, positive sera were absorbed with thick suspensions of all the organisms isolated from a given specimen of saliva. These included a *Streptococcus viridans* and a *Neisseria catarrhalis*. In addition, absorptions were also done with samples of *E. coli*, *Staph. aureus* and *Strep. faecalis*. These results are also summarized in Table 1.

4. *Complement fixation*

All sera were tested against antigen prepared from human heart; some were also tested against extract of human voluntary muscle, human saliva and both types of muscle from rabbit and guinea-pig. The end point was taken as the highest serum dilution giving 50 per cent haemolysis and the positive titres ranged from 1:24 to 1:768.

TABLE 2
RESULTS OF COMPLEMENT FIXATION TESTS

Antiserum	Antigen	MHDs of complement	Titre
Type 2 Group A	Human heart alcoholic extract	5	192
	Human heart saline extract	5	192
	Human voluntary muscle saline extract	5	96
Type 24 Group A	Human heart suspension	3	0
	Human heart alcoholic extract	3	0
	Human heart saline extract	3	0
	Human voluntary muscle saline extract	3	0
	Rabbit voluntary muscle saline extract	3	0
	Guinea-pig voluntary muscle saline extract	3	0
	Guinea-pig heart saline extract	3	0
	Rabbit heart saline extract	3	0
	Human saliva	3	0
Saliva-treated type 24 Group A	Human heart saline extract	5	384
	Human saliva	5	192
	Rabbit heart saline extract	5	48
	Guinea-pig heart saline extract	5	48
	Human voluntary muscle saline extract	5	384
Group D streptococcus	Human heart saline extract	3	0
	Human saliva	3	0
	Rabbit heart saline extract	3	0
Saliva-treated Group D	Human heart saline extract	3	192
	Human saliva	3	96
Human saliva antiserum	Human heart saline extract	3	24
	Human voluntary muscle saline extract	3	48
	Human saliva	3	48
Group G streptococcus	Human heart saline extract	5	192
	Human voluntary muscle saline extract	5	192

Note: (1) Titres are expressed as the reciprocal of the highest serum dilutions showing 50 per cent haemolysis; (2) heart and voluntary muscle suspensions were 1 per cent suspensions of the fine homogenates thereof; and (3) 0 = no fixation.

TABLE 3
RECIPROCAL AGGLUTINATION TITRES OF COATED SHEEP RED CELLS

Antiserum	Human heart saline extract	Human heart alcoholic extract	Human voluntary muscle saline extract	Human saliva
Group A type 6	128	64	64	128
Group A type 19	32	32	64	32
Group A type 24	0	0	0	0
Saliva-treated Group A type 24	256	128	128	256
Group C	0	0	0	0
Saliva-treated Group C	128	128	64	256
Group A type 18	64	128	64	128
Group A type 30	64	64	128	256
Group G	128	64	64	256

Controls are not included in the table as they were all negative.
0 = No agglutination.

The results were remarkably consistent and were in complete agreement with those obtained by immunofluorescence. This applies both to the various organisms used, as well as to the various tissue extracts and to saliva. Some of the results are summarized in Table 2.

5. *Sensitized sheep cell agglutination*

Only selected antisera were used and the results are shown in Table 3. Positive agglutination was obtained with all those sera that were positive by the immunofluorescent test, and conversely. The results were similar with all four varieties of antigen used for coating. Finally, as in the other tests employed, the antisera to the saliva-coated organisms, type 24 and Groups C and D, gave positive results in contrast to the results with the corresponding antisera to the uncoated organisms.

6. *Skin tests*

The test antigens used were human saliva diluted 1:10, and similar dilutions of extracts of human heart, voluntary muscle, liver and spleen. In addition, extracts of three types of streptococci were used, types 2 and 6, known to possess the cross-reacting antigen and type 24, from which the antigen is apparently absent. The reactions were read at 4, 24 and 48 hours. Five rabbits previously immunized with human saliva in Freund's complete adjuvant gave both immediate and delayed type reactions at the sites tested with both cardiac and voluntary muscle extracts, with saliva and with extracts of Group A streptococci types 2 and 6. Negative reactions were obtained with the extracts of liver and spleen, and with the extract of the type 24 streptococcus.

7. *Effect of solvents and enzymes on the cardiac antigen*

This was studied by leaving sections at room temperature in various solvents and testing them for immunofluorescence after thorough removal of the solvent. A potent anti-type 6 antiserum was used throughout. Negative staining was obtained after ethanol, methanol and N/5 HCl. After acetone, ether, Coons's buffer and phosphate buffer, pH 7·8, staining was still strongly positive. That the negative staining was due to extraction, not destruction, of the antigen was indicated by the presence of the antigen in the extracting fluid, as shown by inhibition and complement fixation tests. N/5 alkali destroyed the section. Boiling buffer solutions also extract the antigen from cardiac and voluntary muscle, as shown by inhibition of immunofluorescence and by complement fixation.

Although the antigen as present in cardiac and voluntary muscle was readily extractable by ethanol, the cross-reacting antigen in saliva and in acid extracts of streptococci was completely precipitated by the addition of ethanol to a final concentration of 50 per cent v/v. The antigen in muscle, saliva and in extracts of streptococci was completely inactivated by tryptic digestion after overnight incubation, using 1 mg of crystalline trypsin to each gram of homogenate in the case of muscle, and of alcoholic precipitate in the case of saliva or streptococcal extract.

DISCUSSION

It is evident that our results confirm the original observations of Kaplan and Meyeserian (1962) that some strains of Group A β-haemolytic streptococci contain an antigen that cross-reacts with an antigen in human myocardium. This streptococcal antigen is, however,

of wider distribution than indicated by Kaplan since it was readily detected in nine out of ten types of group A as well as in the single example examined of Group G. Its precise distribution in the micro-organism is unknown but it is distinct from the M protein. From its presence in the glossy form of type 12 and its absence from the L form of the same type, it is most probably located in the cell wall.

Little is as yet known of its chemical nature, and like the ABH blood group substances this seems to differ according to its origin. Thus, in acid extracts of positive streptococci and in saliva it is insoluble in ethanol and completely precipitated at a concentration of 50 per cent v/v. In myocardium and in voluntary muscle it is readily soluble in alcohol and can be easily obtained by this means for complement fixation and other immunological reactions. Like blood group substance, it is present in saliva, but its presence is not influenced by the secretor gene and it could be detected by its ability to inhibit specific immunofluorescence in the saliva of each of forty individuals, irrespective of their secretor status.

It is of interest that no difference was detected in the incidence of the cross-reacting antigen in streptococci, whether they were originally isolated from cases of rheumatic fever or not. Kaplan's original findings indicated a much lower incidence of the antigen in streptococci and a correlation between its presence and the ability to induce rheumatic fever was an attractive possibility. Our own findings, however, imply that the great majority of streptococcal infections involve organisms containing the antigen, and consequently the subsequent development of rheumatic fever must be largely determined by other factors. We considered the possibility that only individuals lacking the antigen would produce the antibody when suffering from an infection with positive streptococci, since those possessing the antigen would presumably be immunologically tolerant to it. But the saliva from a small number of patients known to have had rheumatic fever showed the presence of the antigen in each specimen by inhibition of specific immunofluorescence.

Although the antigen as it exists in the saliva is probably rich in polysaccharide as it is completely precipitated by 50 per cent v/v alcohol, a significant protein moiety is also suggested by its digestibility with trypsin. Moreover the product of trypsin digestion is immunologically inert, possessing neither antigenic nor haptenic properties. Here again the resemblance to a blood group substance is close and suggests the presence of a central protein core to which the other groups are attached as multiple side chains, and which largely lose their immunological specificity when the core protein is disintegrated. This contrasts with the so-called nephrotoxic antigen which on tryptic digestion loses its antigenicity but itself retains its specific haptenic properties (Cole, Cromarty and Watson, 1951).

It is at present impossible to attribute any pathogenic significance to the cross-reacting antigen of streptococci. None of our animals, rabbits or guinea-pigs, showed any evidence of cardiac injury, despite the presence of fairly high titres of antibody. Nor was the situation altered by immunizing the animals with antigen in Freund's complete adjuvant intradermally instead of by the antigen alone intravenously. In view, however, of the widely accepted participation of streptococci in the pathogenesis of rheumatic fever, it would be unwise to regard this organismal antigen as entirely without pathogenic significance.

ACKNOWLEDGMENT

One of us (L.S.N.) was in receipt of a grant from the United Arab Republic.

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