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Study on Streptococcus Group A Antigens Common with Heart Tissue Elements

I. M. LYAMPERT, O. I. VVEDENSKAYA AND T. A. DANILOVA

Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Science, Moscow, U.S.S.R.

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Summary. Four different cross-reactive antigens were revealed in cultures of group A streptococci by immunoelectrophoresis using antistreptococcal sera unabsorbed, or absorbed with homogenates of rabbit heart tissue. Preliminary study of these sera by immunofluorescence showed that they react with different elements of the myocardium.

The hypothesis is suggested that the biological (immunizing) action of microbial cross-reactive antigens depends on the presence or absence of immunological tolerance to these antigens in the individual immunized. The mechanism of autoantibody formation by immunization with such common antigens is discussed.

INTRODUCTION

A common or so-called cross-reactive antigen is present in cell walls of haemolytic streptococci of group A and in the sarcolemma and subsarcolemmal part of the sarcoplasm of human and animal heart tissue myofibres. Antibodies to this antigen were found in complement fixation tests and by the indirect method of immunofluorescence in the sera of rabbits immunized with the cultures of type 5 and type 19 group A streptococci (Kaplan and Meyerserian, 1962; Kaplan, 1963, 1965).

In our studies antibodies reacting in complement fixation tests with extracts of human and animal heart tissue were found in the sera of animals immunized with streptococci of type 1 (Lyampert, Belatzkaya, Borodiyuk and Smirnova, 1962; Lyampert, Galatchyanz, Belatzkaya and Smirnova, 1963), and later antibodies reacting with the sarcolemmal region of myofibres were demonstrated by the indirect immunofluorescent technique in the sera of rabbits immunized with strains of type 5 and type 29 streptococci (Danilova, 1966; Lyampert, Danilova, Borodyuk and Beletzkaya, 1966). The character of these latter reactions was in general similar to that described by Kaplan (1963, 1964). Our type 1 antisera, however, did not react with sarcolemma of myofibres but specifically stained intercalated disks of heart tissue (the elements connecting two myofibre cells of myocardium, absent in skeletal muscle).

By absorbing the antisera with homogenates of heart, skeletal muscle or liver tissues, it was shown that these reactions were specific for heart tissue, in the case of type 1, and for heart and skeletal muscles in the case of type 5. It was also shown that reaction of sera with cardiac tissue was completely eliminated only by absorption with homologous streptococcal organisms. Absorption with heterologous strains did not abolish these reactions.

The data obtained gave evidence that streptococcal cultures types 1 and 5 contain two different cross-reactive antigens (Lyampert *et al.*, 1965).

In the present experiments immunoelectrophoresis was used to reveal different cross-reactive antigens in cultures of group A streptococci. Extracts of streptococcal cultures were examined with streptococcal sera both unabsorbed and absorbed by heart tissue. Such a technique was employed by Kaplan and Svec (1964), to reveal antibodies against a cross-reactive antigen.

To detect antibodies against heart tissue, the sera used were tested by the immunofluorescent method. Only those reactive with sarcolemma and subsarcolemmal region or with intercalated disks of cardiac tissue were employed for immunoelectrophoretic studies.

MATERIALS AND METHODS

Group A strains of haemolytic streptococci containing M protein (type 1 N 2/55, type 5 N 6/55 and type 29 N 15/15) were passed twice through donor's blood to increase M protein content (Becker, 1964). Selected cultures were repeatedly (ten times) passed through casein medium and grown in it.

Antigens for testing in immunoelectrophoresis. HCl extracts prepared according to Lancefield's (1928) procedure were concentrated ten-fold by evaporation under the fan, and dialysed against 0·2 per cent solution of NaCl. The protein antigens were partially purified and separated from the group specific carbohydrate by precipitation with 3 volumes of 96 per cent alcohol, absorption on $Al(OH)_3$ and subsequent elution with 10 per cent solution of sodium citrate (Vvedenskaya and Shishova, 1964). Antigens were dialysed against veronal–medinal buffer solution pH 8·6, before immunoelectrophoresis.

Sera were obtained by immunization of rabbits for 4 weeks with cultures killed by heating (at 56° for 1·5 hours) and thereafter for 2–3 weeks with increasing doses of living cultures.

Repeated cycles of immunization were performed with 1 month's rest between each. Sera were taken after 2–3 cycles of immunization. To prepare sera against type 29, immunization with cell walls was also used (Kaplan, 1963); these were prepared by sonic disintegration with glass beads in a Mickle's disintegrator. Normal rabbit sera were used as controls.

Absorption of sera. To remove group specific antibodies, sera used for immunoelectrophoresis were absorbed with streptococci of type 1, autoclaved for 2 hours at 130°.

To reveal cross-reactive antigens in immunoelectrophoresis, sera were first absorbed with rabbit heart, liver and kidney tissues. Organs were finely minced, repeatedly washed with saline, homogenized and treated with 5 per cent solution of bovine albumin to eliminate non-specific reactions (Kaplan and Svec, 1964). After repeated washing with

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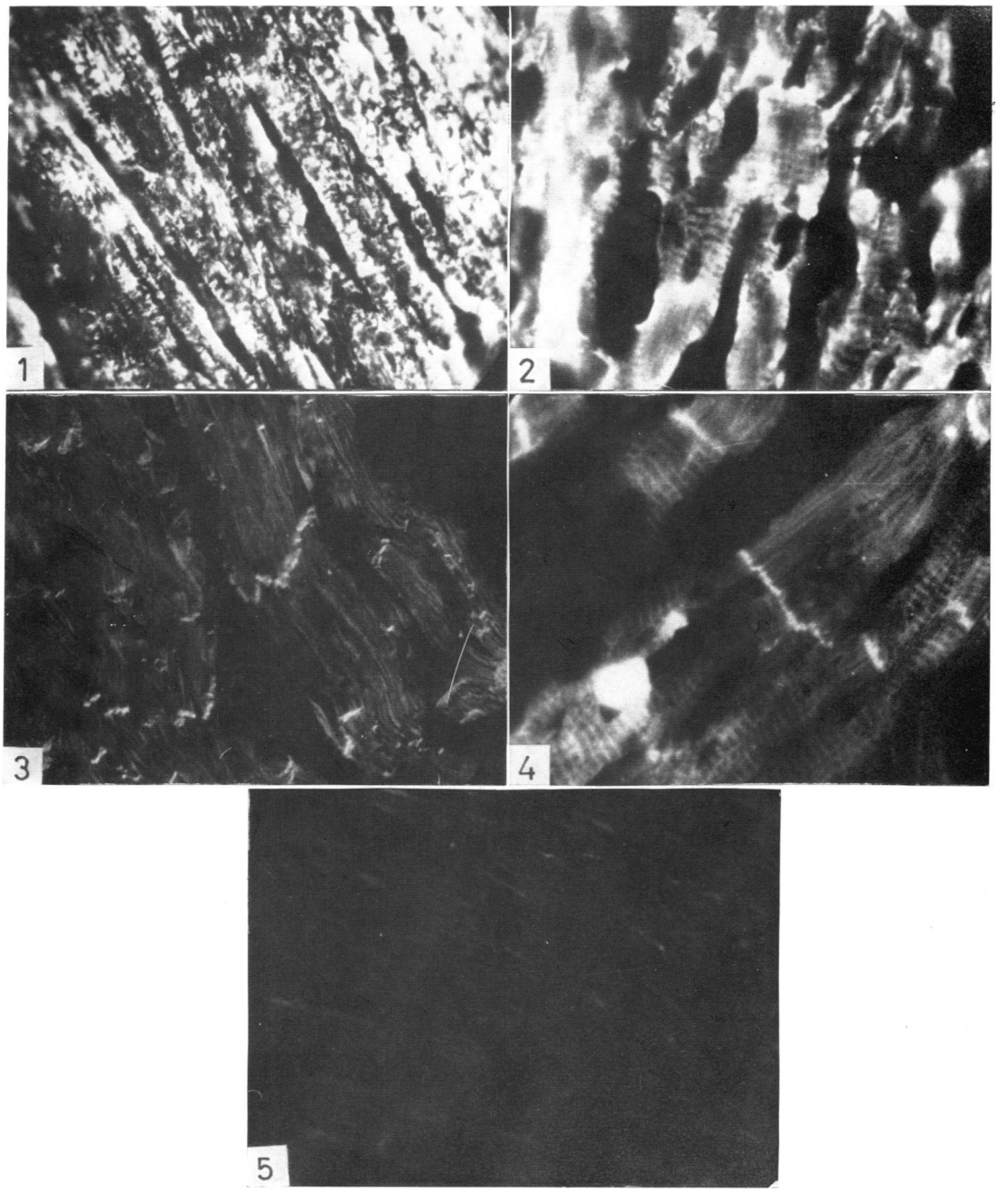
FIG. 1. Type 5 antiserum. Immunofluorescent staining of sarcolemma, subsarcolemma and A-bands in a section of guinea-pig heart tissue. × 40 × 3.

FIG. 2. Type 5 antiserum. Immunofluorescent staining of sarcolemma, subsarcolemma and A-bands in a section of human heart tissue. × 90 × 3.

FIG. 3. Type 1 antiserum. Immunofluorescent staining of intercalated disks in a section of guinea-pig heart tissue. × 40 × 3.

FIG. 4. Type 1 antiserum. Immunofluorescent staining of I-bands and intercalated disks in a section of human heart tissue. × 90 × 3.

FIG. 5. Type 1 serum absorbed with heart tissue. No immunofluorescent staining of section of guinea-pig heart tissue. × 40 × 3.



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saline, 0·1 ml of packed tissue homogenate was used to absorb 0·2 ml of serum. The mixture was incubated for 30 minutes at 37° and overnight in the cold.

The indirect immunofluorescent technique (Weller and Coons, 1954) was used to discover antibodies reacting with heart tissue. A modification of the method employing purified antibodies to rabbit γ-globulin labelled with fluorescein isothiocyanate was used (Engelgardt, 1964). The pure antibodies to γ-globulin were isolated from a goat anti-rabbit serum by an immunosorbent technique (Gourvich, 1964) as follows. Rabbit γ-globulin was obtained from normal rabbit sera by precipitation with 50 per cent ethanol (Zilber and Abelev, 1962) followed by purification in preparative electrophoresis in gel and was conjugated with aminocellulose suspension by diazotation. After treatment with goat anti-rabbit serum, antibodies were eluted from the immunosorbent under acid conditions, and concentrated by evaporation. Concentrated eluates contained 15 mg/ml of protein and gave one precipitin line in gel precipitation with normal rabbit sera.

Antibodies to γ-globulin were conjugated with fluorescein isothiocyanate by the method of Riggs, Seiwald, Burckhalter, Downs and Metcalf (1958) as modified by Blagoveschensky and Kulberg (1962). Excess of dye was removed by filtration through Sephadex G-25. Sections of rabbit heart tissue, 4 μ thick, were prepared in a cryostat at −20°. Unfixed sections were used.

Staining was performed at room temperature for 40 minutes with antistreptococcal sera and 35 minutes with labelled antiglobulin antibodies. Sera were previously twice absorbed with liver powder.

Control experiments. Staining of sections with normal rabbit sera, staining only with labelled antibodies, and inhibition of fluorescence with unlabelled antibodies were performed.

Microscopy. Sections were examined using a fluorescent microscope ML-2 employing exciting filters SZS-7, BS-8, FS-1 and ocular filters GS-18+GZS-19, with an object-glass ×40. To photograph preparations homal ×3 was used.

Methods of obtaining pure antibodies to γ-globulin, preparation of tissue sections and their staining were described in detail in the previous paper (Danilova, 1966).

Immunoelectrophoresis in gel was performed by Grabar's technique (Grabar and Burtin, 1960) with a modification suggested by Abelev and Zvetkov (1960). Agar gel in veronal–medinal buffer, pH 8·6 was used. Electrophoresis was carried out at a potential gradient of 6 V/cm and current of 25 mA for 1 hour at 5°. Pyronin and albumin moved away from the start 2 cm and 1·5 cm respectively after this period of time. The antistreptococcal sera for immunoelectrophoresis were concentrated five-fold by lyophilization and subsequent dissolving in lesser volume.

RESULTS

TESTING OF STREPTOCOCCAL SERA BY THE IMMUNOFLUORESCENT METHOD

Different patterns of reaction were observed when sera were tested by immunofluorescence on heart tissue sections of man, rabbit and guinea-pig. When sections were treated with type 5 sera, bright specific staining was seen in sarcolemmal and subsarcolemmal regions of the sarcoplasm and in some sections in the A-bands of myofibres (Figs. 1 and 2).

Antisera against type 1 gave specific staining in the region of the intercalated disks of myofibres (Fig. 3). In some instances these sera reacted also with I-bands of myofibres in

sections of human heart tissue (Fig. 4). However, staining of I-bands was also observed in human heart sections when sera from non-immunized rabbits were used. Type 29 sera stained the sarcolemmal region of myofibres in the same way as type 5 sera, but their reactions were less bright. Normal rabbit sera did not react with heart tissue with the exception of the above-mentioned reactions with I-bands.

Absorption of type 1 and type 5 sera with homogenates of cardiac tissue abolished the reactions of these sera with the heart tissue sections (Fig. 5).

CROSS-REACTIVE ANTIGENS REVEALED BY IMMUNOELECTROPHORESIS

The extracts obtained from streptococci of type 1 gave two precipitin lines when tested by immunoelectrophoresis with non-absorbed sera. Both lines were reactions due to type-specific substances since they were not obtained with sera against heterologous types. These reactions with type 1 sera were not abolished after removal of group specific antibodies. Absorption of antisera to type 1 by homogenates of heart tissue completely abolished the reaction with one of the substances. Absorption by homogenates of the liver or kidney tissues had no effect on the results (Fig. 6). Five antigenic substances were revealed when extracts obtained from streptococci of type 29 were examined by this method, using antisera against the same type. Similar results were obtained when these antigens were tested against sera of rabbits immunized with cell walls.

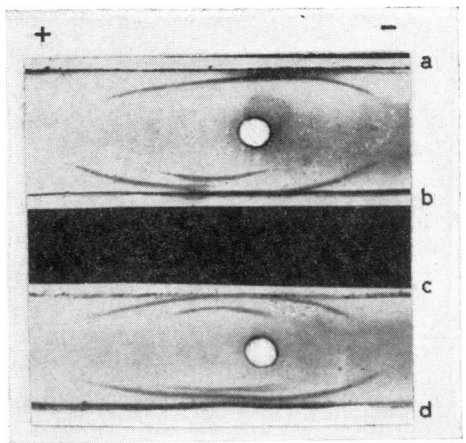

FIG. 6. Reservoir: type I extract. Trench (a) type I serum, absorbed with heart tissue; (b) type I serum, absorbed with liver tissue; (c) type I serum, unabsorbed; (d) type I serum, absorbed with kidney tissue.

The antigens obtained from type 29 did not react with type 1 antisera, and only one of them reacted with serum against type 5. Type 29 serum absorbed with heart tissue homogenate reacted with only two homologous antigens, the antibodies to the three other homologous antigens being absorbed (Fig. 7). The homogenates of liver or kidney tissues did not absorb these three antibodies.

By studying the reactions of extracts of type 5 streptococci with homologous antisera, four antigens were detected. None of these reacted with type 1 sera after removal of group specific antibodies. One of them reacted with type 29 antisera. It was possible to absorb

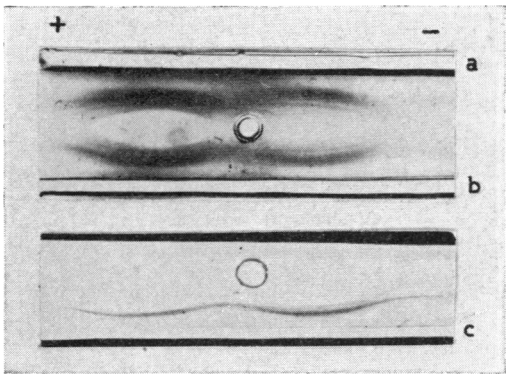

FIG. 7. Reservoir: type 29 extract. Trench (a) type 29 serum, unabsorbed; (b) type 29 serum, absorbed with liver tissue; (c) type 29 serum, absorbed with heart tissue.

antibodies to this antigen with heart tissue but not with liver or kidney homogenates (Fig. 8).

A second type of antigen localized in the area where usually pyronin is revealed was apparently the group C-polysaccharide. This substance could not be digested by trypsin and did not react with the sera after removing group specific antibodies. The other antigens and all antigens revealed in the streptococci types 1 and 29, are apparently proteins since they are digested by trypsin.

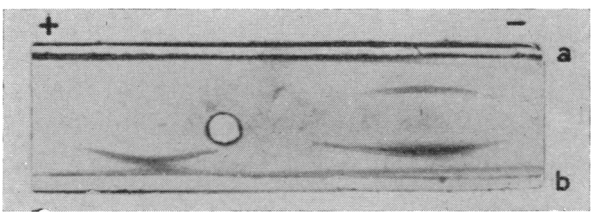

FIG. 8. Reservoir: type 5 extract. Trench (a) type 29 serum, absorbed with heart tissue; (b) type 29 serum, absorbed with liver tissue.

DISCUSSION

Four antigenically different substances were detected in the tested cultures, which did not react with the type antisera after the latter had been absorbed with the heart tissue. Three antigens were present in type 29 sera and one in type 1 sera. Absorption by heart tissue apparently removed only specific antibodies since absorption with liver or kidney homogenates had no influence on the results of the reactions. Therefore all four substances can apparently be considered as cross-reactive antigens which are immunologically related to some elements of heart tissue. These cross-reactive antigens are proteins. Three of them revealed in streptococci of type 29 are antigens of the cell wall, since they reacted with the antisera made against cell walls. One of the substances discovered in type 29 appears to be common with the cross-reactive antigen of type 5. This cross-reactive antigen

might be supposed to correspond to certain elements of myofibre sarcolemma or subsarcolemmal sarcoplasm, because by immunofluorescence these sera reacted with these areas in heart sections. Furthermore, this antigen had the same electrophoretic mobility as the first cross-reactive antigen detected by Kaplan and Svec (1964).

The type 1 sera, judging by the results obtained by immunofluorescent test, reacted with the intercalated disks. In immunoelectrophoresis tests these sera reacted only with antigens obtained from type 1 but not with those obtained from types 5 or 29. In the light of these findings the cross-reactive antigen detected in the type 1 strain (antigen N 2) apparently differs from the above antigen found in the streptococcal types 5 and 29. Mention should also be made of the fact that the anti-type 5 antisera reacted with A-bands, and the anti-type 1 antisera (and sera of some normal rabbits) reacted with I-bands of myofibres. These reactions are very similar to those obtained with sera from myasthenia gravis patients and from some healthy persons (Beutner, Witebsky and Djanian, 1965; Vetters, 1965).

Kaplan (1964) reported data testifying the formation of antibodies to the first cross-reactive antigen in patients suffering from rheumatic fever. However, a reaction in the region of the sarcolemma of myofibres is also obtained with sera of patients with myocardial infarction and the sera of patients after commissurotomy (Kaplan, Meyeserian and Kushner, 1961; Van der Geld, 1964). It thus appears that the question of the specificity for rheumatic fever of antibodies arising against the first cross-reactive antigen is far from being solved.

It is likely that the presence or the absence of immunological tolerance in an organism determines the nature of the biological action of the cross-reactive antigens present in a number of micro-organisms (see Table 1). When some micro-organisms have antigens corresponding to isoantigens, antibodies could be produced by the individuals lacking the same antigen. But in individuals possessing the same isoantigens, microbial cross-reactive antigens are recognized as 'self'. Microbial cells with this antigen on their surface might not be phagocytosed and may therefore be more virulent.

A similar case was described by Rowley and Jenkin (1962). Cross-reactive antigen recognized as 'self' but localized inside the bacterial cell apparently has little effect on virulence. When the cross-reactive antigens correspond to organ-specific components of tissues to which there is immunological tolerance, their biological action is also due

TABLE 1

SCHEME OF BIOLOGICAL ACTION OF CROSS-REACTIVE ANTIGENS OF MICRO-ORGANISMS

Tissue elements corresponding to cross-reactive antigens of micro-organism	Presence or absence of cross-reactive antigens in the tissues of individuals	Immunological tolerance to these antigens	Localization of cross-reactive antigens in micro-organisms	Effect of antigen
Isoantigens	Absent	–	On the surface or inside a micro-organism	Antibody production
Isoantigens	Present	+	On the surface Inside	Virulence increase Absence of action
Organ-specific antigens	Present	+	On the surface Inside	Virulence increase Absence of action
		–	On the surface or inside	Formation of antibodies reacting with the tissue-autoantibodies

to their localization in their cell (see Table 1). Quite different, however, must be the biological effect of cross-reactive antigens of micro-organisms which are common with organ-specific components to which there is no immunological tolerance, or to which tolerance is weak. In this case, one might expect formation of antibodies reacting with the corresponding tissue components. The first cross-reactive antigen discovered by Kaplan *et al.* (1962, 1963) and the second described in our papers correspond, apparently, to organ-specific tissue components.

Such tissue components can be demonstrated in the heart tissues of man and of animals of different species (Danilova, 1965; Lyampert *et al.*, 1965), and antibodies to these elements can be produced by immunization with streptococci containing cross-reactive antigens. This phenomenon testifies to the absence or insufficiency of immunological tolerance to the corresponding tissue components or termination of tolerance. The immunizing action of the cross-reactive antigens of streptococcus is presumably due to the presence in the micro-organism of antigens which, although foreign, share determinants in common with the host tissues, and it provides a parallel to the termination of tolerance towards such common determinants described by Weigle (1965). In Weigle's investigations acquired immunological tolerance to serum proteins was terminated when the same protein conjugated with chemicals was injected. The presence of determinants which can be recognized as 'foreign' promotes the termination of tolerance to the 'self' determinants. Witebsky (1965) also suggested that, when immunization is performed with heterologous tissues, the immunizing action of organ-specific antigens common for different species is due to the presence in tissues of antigenic determinants foreign to, as well as common to, the immunized species.

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