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IMMUNOLOGICAL AND BIOLOGICAL NATURE OF ANTIGENS OF STREPTOCOCCUS MITIS AND STREPTOCOCCUS SALIVARIUS

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ABSTRACT

Extracellular antigens have been isolated from *Streptococcus mitis*, *Streptococcus salivarius* and group A streptococcal cultures grown in a synthetic medium. Analysis of the antigens was performed by immunoelectrophoretic and double diffusion techniques using rabbit immune sera. *S. mitis* cultures produced 10 antigens, *S. salivarius* six antigens and group A streptococcus 12 antigens, when tested with their corresponding antisera. *S. mitis* and *S. salivarius* antigens had only one common antigen when tested with antisera to both antigen pools. No cross reaction was found between the exo-antigens of group A and viridans streptococci. While the extracellular antigen pool of group A streptococci contained ribonuclease, deoxyribonuclease, hyaluronidase, diphosphopyridine-nucleotidase, streptokinase and streptolysin O, that of *S. mitis* contained only ribonuclease and that of *S. salivarius* contained hyaluronidase and collagenase. Each of the three streptococcal antigen pools contained a hemosensitizing factor which sensitized mammalian cells to passive immune kill. Sonicates produced from the mitis, salivarius and group A streptococcus contained six antigens, most of which cross reacted with each other. *S. mitis* sonicates were separated into six fractions by ion exchange chromatography on ECTEOLA cellulose, and into three major fractions following gel-filtration on Sephadex G-200 columns.

Rabbits injected i.v. with sonicates derived from *S. mitis* developed cardiac and hepatic lesions which, in some cases, were accompanied by a steep rise in serum glutamic oxalacetic transaminase, sorbitol dehydrogenase and total lipids. The relationship of tissue damage to enzyme rise is discussed in relation to the possible early diagnosis of tissue damage following streptococcal infection.

INTRODUCTION

Streptococci belonging to the viridans group are known as etiological agents in the pathogenesis of subacute bacterial endocarditis and of dental caries in both man and laboratory animals (1–3). Little is known about the antigenic composition and the biological properties of the extracellular and intracellular products derived from these organisms.

In this paper we present an immunological analysis of some of the antigens of *S. mitis*

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and *S. salivarius*, and give a preliminary report on the tissue changes in rabbits injected with sonicates derived from *S. mitis*.

MATERIALS AND METHODS

Microorganisms. S. mitis and *S. salivarius* strains were isolated from human saliva by growing the organisms on a mitis–salivarius agar (Difco).

Group A hemolytic streptococci (type 6) were obtained from the Streptococcus Reference Laboratory, Ministry of Health, Jerusalem. All organisms were kept on blood agar slants.

Streptococcal antigens. a) *Extracellular. S. mitis* and *S. salivarius* were cultivated at 37 C in 5 liter batches of a synthetic medium previously described by Ginsburg and Grossowicz (4). Acid produced by the streptococci during growth was neutralized every 30 min by 1 N NaOH. At the end of the logarithmic phase of growth the supernatant fluids obtained following centrifugation at 10,000 $\times g$ with a refrigerated centrifuge, were dialyzed overnight at 4 C against 0.02 M phosphate buffer pH 7.4, concentrated 10-fold by pervaporation and saturated with ammonium sulfate.

The resulting precipitate was collected by centrifugation at 36,000 $\times g$ for 10 min. It was dissolved in distilled water and desalted by passing through a Sephadex G-25 column (30 $\times$ 1.8 cm) (Pharmacia, Uppsala, Sweden) previously washed with 0.01 M phosphate buffer pH 7.4. The material was further concentrated by pervaporation and kept at –25 C. The extracellular products derived from *S. mitis* and from *S. salivarius* were designated as EXP-M and EXP-S respectively.

Extracellular antigens from a group A streptococcus (type 4) were obtained in a similar way from cultures grown in a completely synthetic medium in a chemostat (5). These antigens were designated as EXP-A.

b) *Cellular antigens* were obtained from all streptococci following sonification in a 9 Kc Raytheon sonic oscillator for 90 min. The bacterial debris was removed by centrifugation at 36,000 $\times g$ with a refrigerated centrifuge; the supernatant fluid obtained was concentrated by pervaporation, dialyzed against distilled water at 4 C, filtered through a millipore filter (0.45 μ) and kept at –25 C.

Production of antisera. Antisera to EXP-A, EXP-M and EXP-S and to the sonicates of all bacteria were obtained following immunization of rabbits with antigens in complete Freund's adjuvant. Two to three weekly injections of 2 mg of antigens were given for three weeks and the animals were bled one week following the last injection.

Immunological methods. Rabbit antisera to the various antigens were analyzed by double diffusion and immunoelectrophoretic techniques according to established procedures. Passive hemagglutination with the extracellular and intracellular antigens were performed according to the method described by Dishon et al. (6).

Ion exchange chromatography. Separation and isolation of streptococcal antigens was achieved by ion exchange chromatography on ECTEOLA cellulose (a reaction product of epichlorohydrin, triethanolamine and cellulose) (Whatman Co.) by both stepwise and gradient salt elution techniques, as described by Ginsburg and Harris (7).

Analytical methods. Protein was determined by the method of Lowry et al. (8) using human serum albumin and EXP-A as standards. Determination of protein was also performed by measuring the absorption at 280 μ with a Hitachi Model 190 spectrophotometer. Rhamnose was determined according to the method of Dische and Shettles (9).

Ribonucleic acid (RNA) was determined by the Orcinol method (10) or by determining the absorption at 260 μ. Deoxyribonucleic acid (DNA) was determined by the diphenylamine test (10).

Determination of serum enzymes and total lipids. Prior to the experiments and 24 hr following the injection of the streptococcal products (see below), the sera of the animals were analyzed for their content of glutamic oxalacetic transaminase (GOT), by the method of Reitman and Frankel (11) and for sorbitol dehydrogenase (SOD) as described by King (12). Total lipids were determined by the method of Dole (13).

Determination of streptococcal enzymes. The extracellular antigens and sonicates derived from the streptococci were analyzed for the presence of streptolysin O (SLO), hyaluronidase (Hydase), deoxyribonuclease, ribonuclease, diphosphopyridine nucleotidase and proteinase according to the methods described by Kwapinski and Snyder (14). Collagenase was determined by a modified method of Gross (15).

Animals. Male and female rabbits weighing 2 to 3 kg and Swiss mice weighing 20 g were kept on a purina pellet diet and water ad lib. The streptococcal products were injected into the tonsils as described previously (16).

The animals were sacrificed by an i.v. injection of sodium pentothal. Sections from all parts of

the heart, as well as samples from liver, kidney, spleen, diaphragm and tonsils were fixed in 4% neutral formalin. The tissues were embedded in paraffin and 6 μ sections were stained with hematoxylin and eosin.

RESULTS

Extracellular and cellular antigens of streptococci.

a) *Extracellular.* Under standard conditions 1 liter of *S. mitis* cultures yielded approximately 7 mg of extracellular proteins, *S. salivarius* yielded 3 mg of protein and group A streptococci yielded 15 mg of protein. Immunoelectrophoretic analysis of EXP-M, EXP-S and EXP-A using their corresponding rabbit antisera is shown in Fig. 1. It can be seen that antiserum to EXP-M (Fig. 1a) showed the presence of at least 10 precipitating lines, six of which were negatively charged. Antiserum to EXP-S (Fig 1b) showed six precipitating lines, of which three migrated to the anode, while antiserum to EXP-A (Fig 1c) showed at least 12 precipitating lines, of which eight migrated to the anode. It was also found that antiserum to EXP-M did not react with EXP-A. Similarly antiserum to EXP-A did not react with EXP-M (not shown).

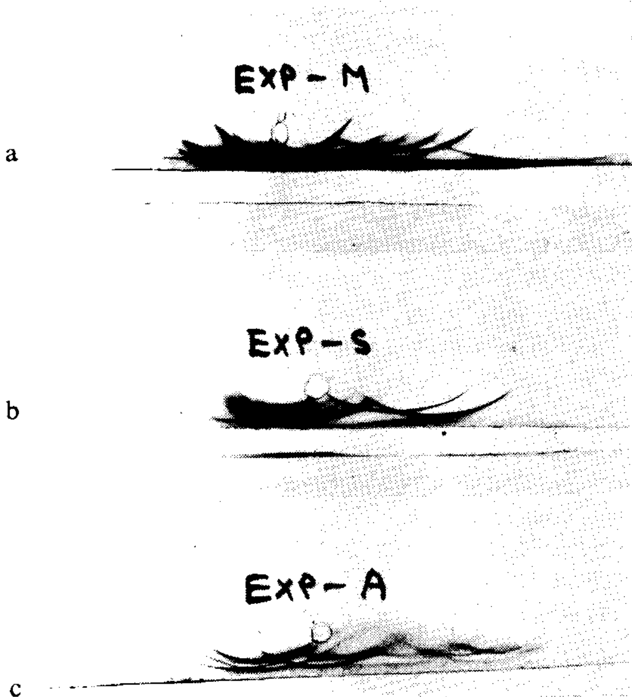
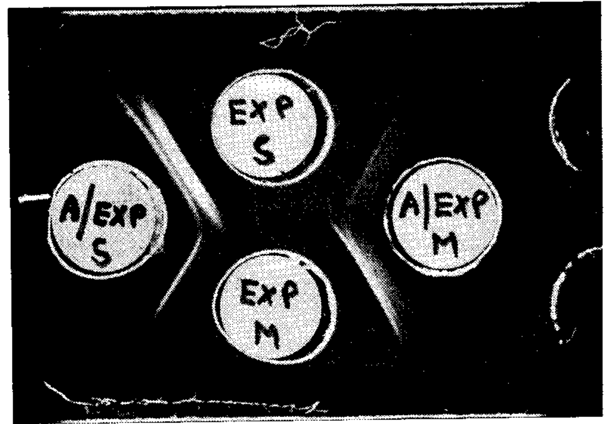


FIG. 1. Immunoelectrophoretic analysis of EXP-M(a) EXP-S(b) and EXP-A(c) with their corresponding rabbit antisera.



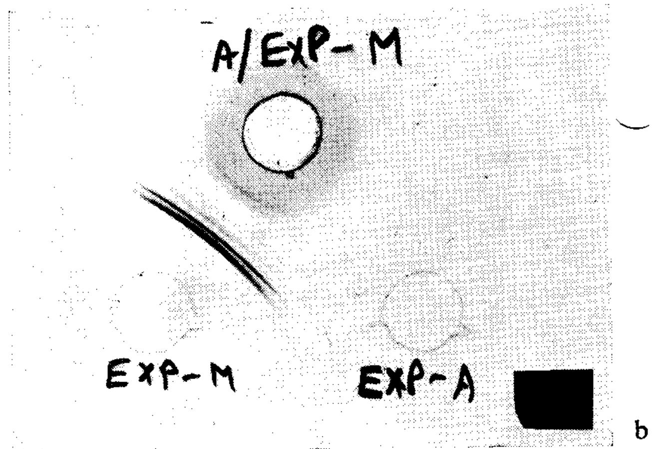


FIG. 2. Double diffusion analysis (Ouchterlony's method) of EXP-M, EXP-S and EXP-A with antiserum to EXP-M(A/EXP-M) and anti EXP-S(A/EXP-S).

Double diffusion experiments were performed to test whether EXP-M, EXP-S and EXP-A had any common antigens. Fig. 2a shows that while both anti-EXP-M and anti-EXP-S sera had several precipitating antibodies when tested with their corresponding antigens, only one common antigen was present.

Comparison of the antigens present in EXP-M and EXP-A are shown in Fig 2b.

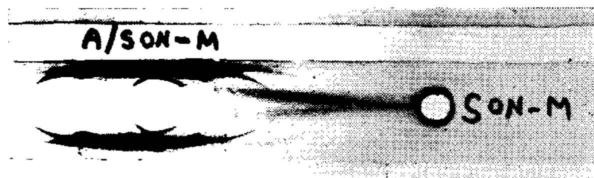


FIG. 3. Immunoelectrophoretic analysis of a sonicate preparation of *S. mitis* (SON-M) with its rabbit antiserum (A/SON-M).

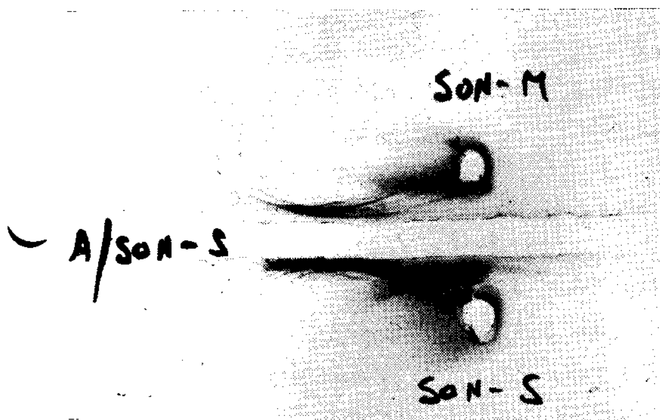


FIG. 4. Immunoelectrophoretic analysis of sonicates of *S. mitis* (SON-M) and *S. salivarius* (SON-S) with antiserum to a sonicate preparation of *S. salivarius* (A SON-S).

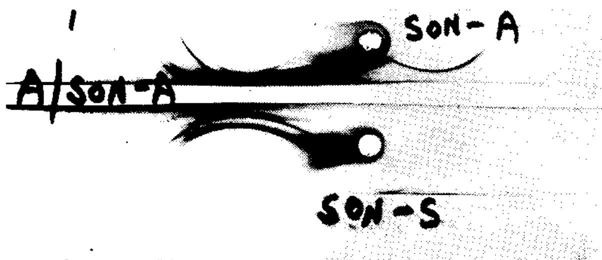


FIG. 5. Immunoelectrophoretic analysis of sonicates derived from group A streptococci (SON-A) and of *S. salivarius* (SON-S) using antiserum to group A streptococcal sonicate (A/SON-A).

While EXP-M gave several precipitating lines with its homologous antiserum, no precipitating lines were obtained with EXP-A. When anti-EXP-A serum was tested with EXP-M erratic results were obtained. One to two precipitating lines appeared in some experiments while in others (using antiserum from other immunized rabbits) no such results were obtained.

EXP-A was previously found to contain hydase, deoxyribonuclease, ribonuclease, diphosphopyridine nucleotidase, SLO and proteinase (17). A search was made for similar enzymes in EXP-M and EXP-S. It was found that EXP-M contained only small amounts of ribonuclease while EXP-S contained hydase and collagenase. None of the other enzymes found in EXP-A were found in any of the preparations of EXP-M or EXP-S. EXP-S, EXP-M and EXP-A all contained a thermostable cell-sensitizing substance capable of sensitizing mammalian cells to passive immune kill (6).

b) *Cellular antigens.* Immunoelectrophoretic analysis of the various streptococcal sonicates using their corresponding antisera is shown in Fig. 3, 4 and 5.

As can be seen, antiserum to *S. mitis* sonicates tested with mitis sonicate possessed at least six precipitating lines, of which five migrated to the cathode (Fig. 3). Antiserum to *S. salivarius* possessed six precipitating lines, most of which appeared similar to antigens present in *S. mitis* sonicates (Fig. 4). Antiserum to group A sonicates also reacted with sonicates of *S. salivarius* (Fig. 5) and *S. mitis* (not shown). Many of the antigens present in the sonicates of the viridans streptococci cross reacted (Fig. 6). There was similar cross reaction between the sonicates of group A and the viridans group (not shown).

Fractionation of S. mitis *sonicates on Ecteola cellulose.* Sonicate preparations containing 30 mg protein and 500 µg of rhamnose were applied on Ecteola cellulose columns washed with 0.01 M phosphate buffer pH 7.2. The material was eluted with a linear salt gradient as described previously (7).

Fig. 7 shows that *S. mitis* sonicates can be separated into six fractions. Peak A emerged from the column with the void volume at 0.01 M phosphate and contained noncharged

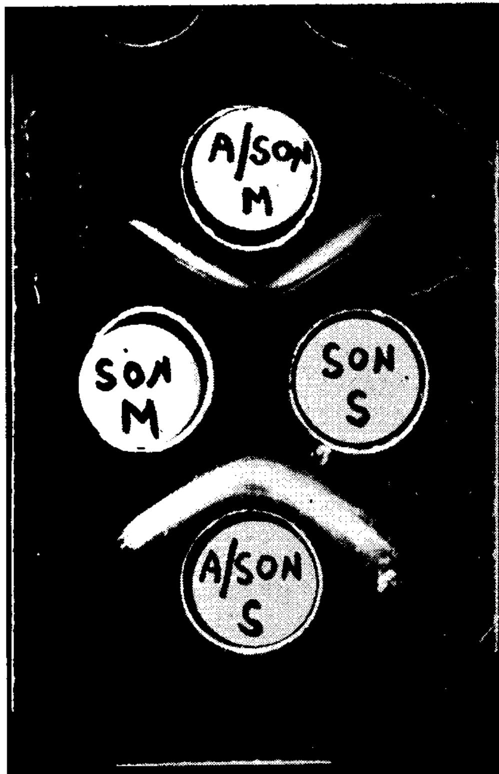


FIG. 6. Double diffusion analysis in agar of sonicates derived from *S. mitis* and *S. salivarius* with their corresponding antisera.

and positively charged material. Peak B came off the column between 0.1 and 0.15 M of NaCl, peak C emerged from the column at approximately 0.2 M of NaCl, peak D appeared at 0.25 M of NaCl, peak E between 0.3 and 0.4 M and peak F between 0.4 and 0.5 M NaCl.

Comparison of the absorbancy at 260 mμ and 280 mμ showed that the components in peaks A, E, F had a higher absorbancy at 260 mμ while peaks B, C, D absorbed ultraviolet higher at 280 mμ. These results pointed to the presence of nucleic acid in peaks A, E, F. Quantitative estimations had indeed shown that RNA was located in peaks A, E, F, while DNA was found only in peak F.

Immunoelectrophoretic analysis of the antigens present in peaks A to F (not shown) revealed that peak A contained one weak antigen. Peak B contains five to six antigens, the majority of which migrated to the anode.

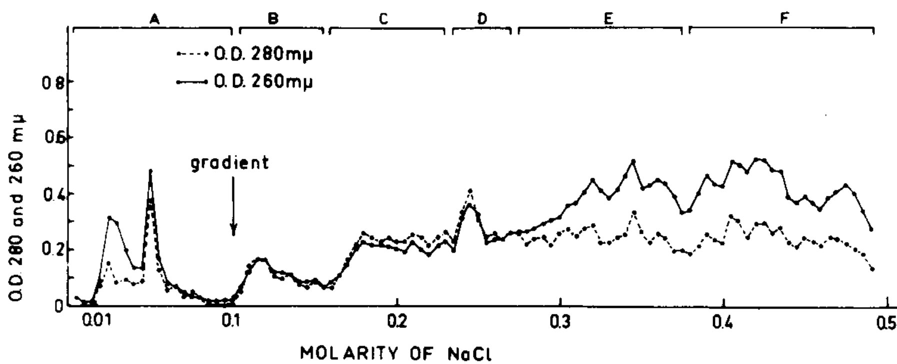


FIG. 7. Gradient salt elution chromatography of *S. mitis* sonicate on Ecteola cellulose.

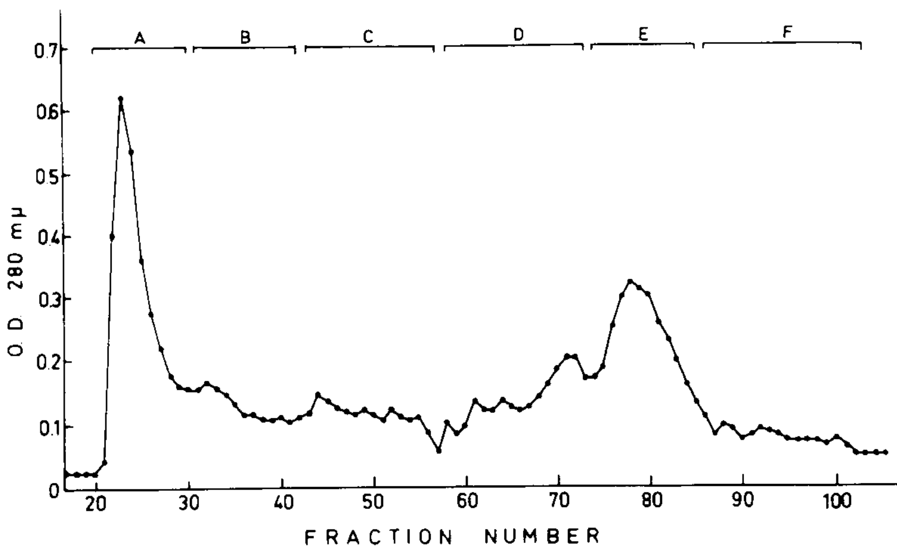


FIG. 8. Gel-filtration of *S. mitis* sonicates on Sephadex G-200 column.

It contained some antigens common with those found in peak C. Peak D contained six antigens, two of which also appeared in peak C. Peak E contained four antigens with a higher rate of migration and peak F contained only one antigen.

Fractionation of S. mitis *sonicates on Sephadex G-200.* One ml of a sonicate preparation containing 25 mg of protein and 400 μg of rhamnose was filtered through Sephadex G-200 column (60 × 3.0 cm). The elutent was 0.15 M NaCl in 0.01 M phosphate pH 7.4. Fig. 8 shows that multiple peaks were obtained (A to F). The material in the different peaks A to F was pooled, concentrated by pervaporation and analyzed for the presence of antigens by immunoelectrophoresis using anti-sonicate M serum. It was found (not shown) that peak A contained three negatively charged antigens, and peak B contained three negatively charged antigens, only one of which seemed common with one of the antigens in peak A. Pool C contained four antigens, three of which were negatively charged and one positively charged. Peak D did not contain any antigens while peaks E to F each contained one positively charged antigen which seemed to be similar.

Toxicity of streptococcal sonicates in rabbits. As shown previously, group A streptococcal sonicates induced nodular skin lesions in rabbits (18), caused cardiac lesions in mice (19) and induced cardiac and hepatic lesions in rabbits (17). To test the specificity of the effect of group A sonicates, four rabbits were injected intratonsillarly with sonicates derived from *S. mitis.* The sonicate preparation injected contained 75 mg of protein and 1,000 μg of rhamnose; three rabbits injected with 75 mg of human albumin served as controls. All the animals were sacrificed seven days following the injection. Table 1 shows that three of the rabbits injected with *S. mitis* sonicates developed cardiac lesions. The lesions comprised multiple areas of myofiber necrosis which were infiltrated with mononuclear cells and histiocytes (Fig. 9). In some cases, calcification of the necrotic muscle fibers was apparent. Three animals developed liver lesions which ranged from coagulation necrosis (Fig. 10) to giant cell granulomas containing multinucleated giant cells which had engulfed amorphous basophilic substance containing calcium (Fig. 11).

Both the cardiac and hepatic lesions were essentially similar to those induced in rabbits by sonicates derived from group A streptococci (17) and by group A extracellular antigens (16). Two animals injected with *S. mitis* sonicates had elevated SGOT, two had elevated SOD, and two had high total lipids 24 hr following injection (Table 1). None of the

TABLE 1. *Toxic effects of* S. mitis *sonicates in rabbits*

No. of animal	*Material injected*	*Lesions in*[a]		*Titers of*[b]		
		Heart	*Liver*	*GOT (IU/ml)*	*SOD (IU/ml)*	*TL (mg%)*
1	Sonicate	N	CN	120	76	1070
2	Sonicate	N	CN GR	96	30	687
3	Sonicate	—	CN	10	6	408
4	Sonicate	N	—	17	3	450
5	Human albumin	—	—	10	4	420
6	Human albumin	—	—	12	5	405
7	Human albumin	—	—	9	6	430

[a] N = Necrosis with moderate inflammatory exudate.
CN = Coagulation necrosis.
GR = Granulomatous inflammation.
[b] Titer of enzyme or total lipids (TL) 24 hr following injection. Average levels of GOT, SOD and TL prior to injection were 11 I.U., 5 I.U. and 420 mg% respectively.

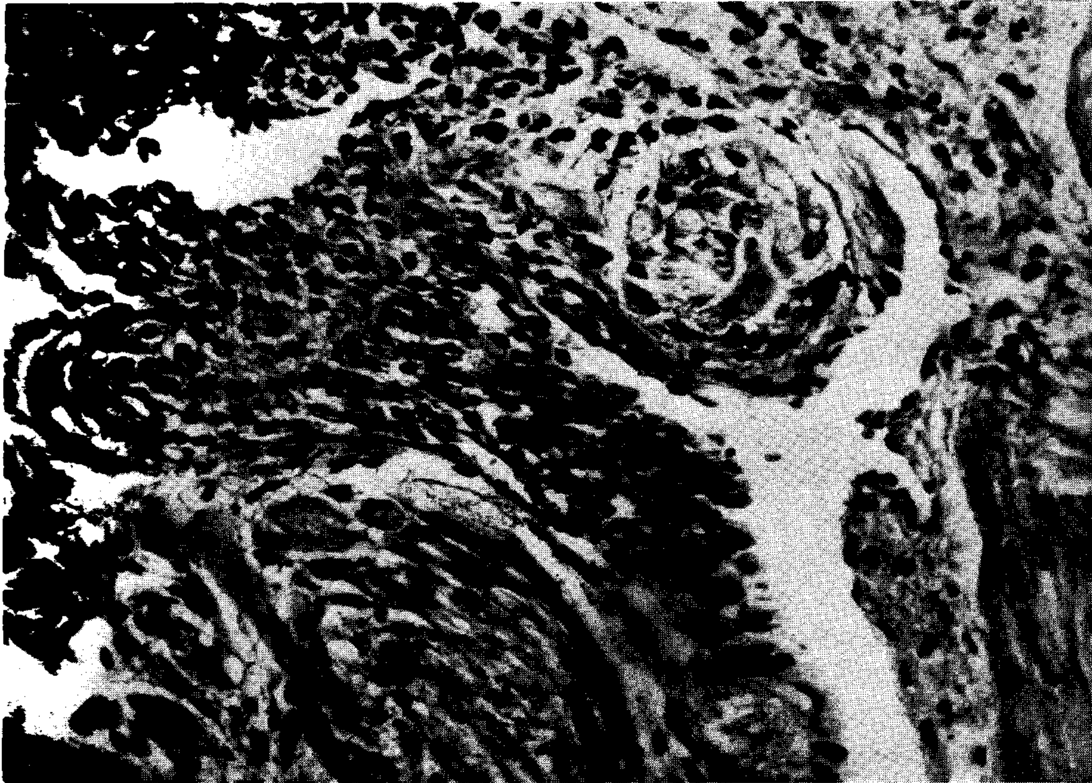


FIG. 9. Myocardial lesion in a rabbit seven days following an intratonsillar injection of *S. mitis* sonicate containing 75 mg of protein and 1,000 mg of rhamnose. × 270.

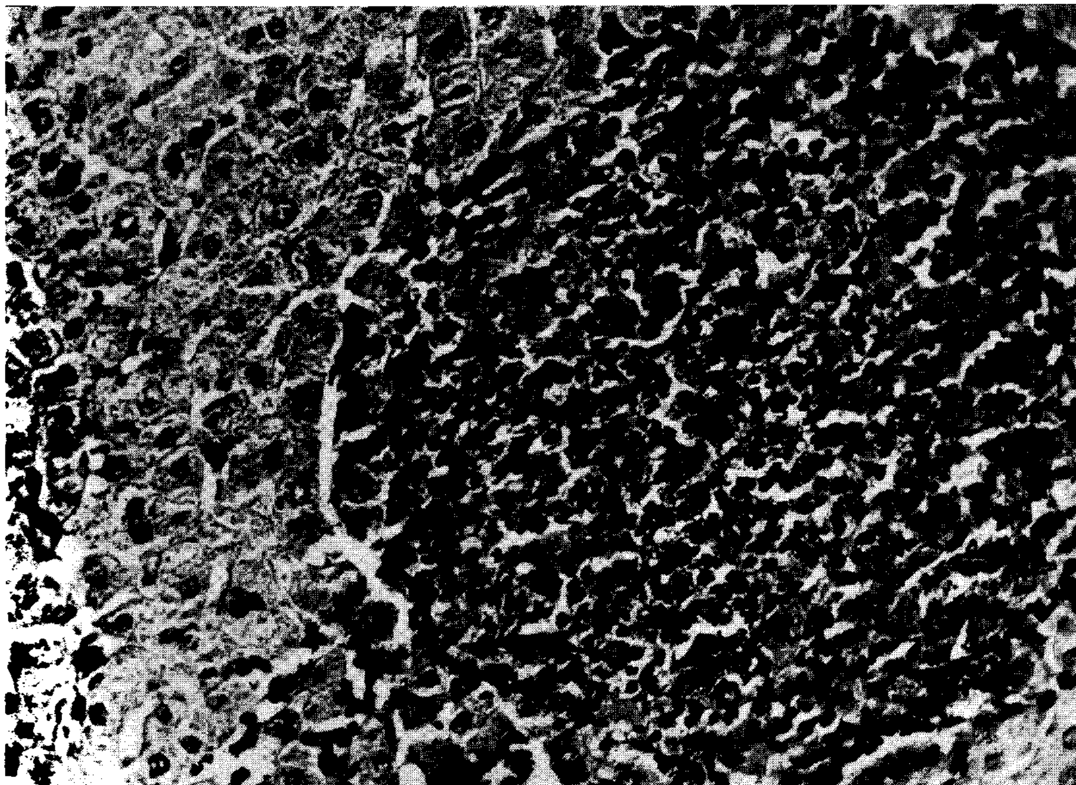


FIG. 10. Hepatic lesion in a rabbit seven days following an intratonsillar injection of *S. mitis* sonicate. Note the area of necrosis and infiltration with granulocytes and mononuclear cells. × 270.

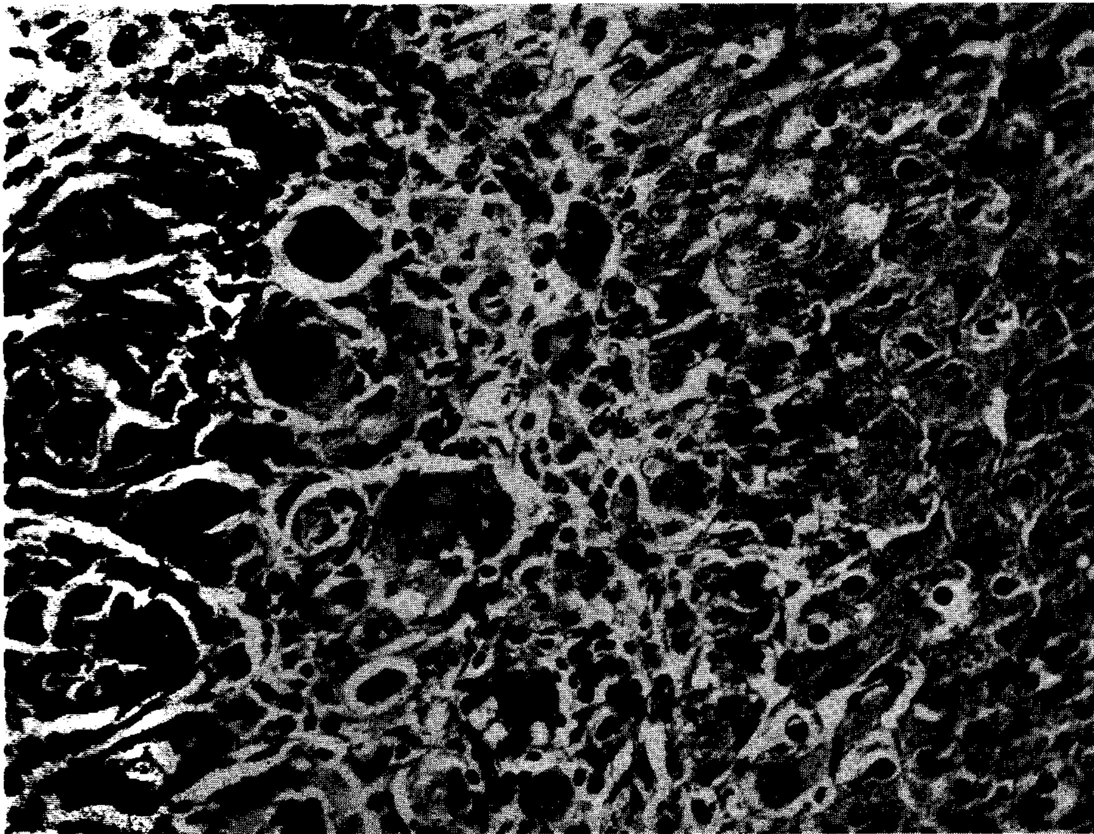


FIG. 11. Hepatic lesion in a rabbit seven days following an intratonsillar injection of *S. mitis* sonicate. Note the giant cell granulomas and foci of calcified clumps. ×270.

animals injected with albumin had any tissue alterations or rise in enzymes.

DISCUSSION

The data presented show that *S. mitis*, *S. salivarius* and group A streptococci produce many extracellular antigens during growth in a synthetic medium (Fig. 1). Many of the antigens are negatively charged. Comparison between the antigens of *S. mitis* and *S. salivarius* shows that only one to two common antigens were present (Fig. 2a). Moreover, none of the numerous exo-antigens produced by group A streptococci (Fig. 1c) cross-reacted with any of the exo-antigens of the viridans group (Fig. 2b). Much is known today about the antigens of group A streptococci, their biological properties and role in human disease (16, 20). On the other hand, only few exo-products of *S. viridans* have been defined. Some viridans streptococci have been shown to produce hyaluronidase (21).

As shown above, EXP-S contained hyaluronidase and small amounts of a collagenase, while EXP-M possessed small amounts of ribonuclease. Both group A streptococci and the viridans group were found to produce a thermostable hemosensitizing factor capable of sensitizing a variety of mammalian cells to passive immune kill (6). The hemosensitizing factors of the two streptococcal species cross-reacted immunologically but also possessed species specific antigenic determinants (6). None of the viridans streptococcal strains tested produced any of the other enzymes and exo-products so characteristic of group A and C streptococci (20). Because of the implication that the viridans group was involved in

the pathogenesis of dental caries it would be of interest to look further for some biological activities of the exo-antigens of this bacterial group.

In contrast to the nonidentity of the exo-antigens among the various streptococci, analysis of the antigens present in sonicates derived from group A and viridans showed many common products (Fig. 6). Most of the sonicate antigens were positively charged. These findings are in agreement with the concept that gram positive microorganisms have similar cell wall components (22).

Sonicates derived from group A streptococci and from a variety of gram positive organisms have been shown to possess distinct biological properties (17, 18, 23). Group A streptococcal sonicates have been shown to cause nodular skin lesions in rabbits (18, 19), to induce cardiac and hepatic lesions in rabbits (17) and cardiac and arteritic lesions in mice (19). The data presented above show that sonicates of *S. mitis* caused myocarditis and granulomatous lesions in livers of rabbits which are indistinguishable morphologically from those caused by group A sonicates. Further study on the nature of the toxic factors is in progress.

The appearance of tissue damage in the animals was accompanied, in two animals, by a rise in the levels of GOT and SOD which was apparent 24 hr following administration of the sonicates. It is possible that the early determination of enzymes may prove useful for the detection of tissue damage following streptococcal infections (16). The rise in total lipids following administration of sonicates is similar to that found in rabbits following the injection of group A streptococci (16, 17). The reason for the rise in lipids is still obscure. It is possible that this is a secondary reaction to changes caused by streptococcal components. It was recently shown that rabbits receiving i.v. injections of papain also show a rapid rise in total lipids (24).

Finally, the separation between positively and negatively charged antigens by ion exchange chromatography and gel filtration (Fig. 7, 8) and the localization of biologically active components in the various fractions will contribute to the understanding of the role of streptococcal products in human disease.

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