1-HB was devoid of activity in the microbial system.

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Toxic Effects Induced in Rabbits by Extracellular Products and Sonicates of Group A Streptococci* (32908)

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It has been previously shown (1, 2) that myocardial, hepatic, and diaphragmatic lesions were induced in rabbits following the intratonsillar injection of living group A streptococci or of some streptococcal extracellular products (SEP). The myocardial lesions were characterized by muscle necrosis and by infiltration with inflammatory cells. In some cases giant-cell granulomas containing clumps of calcified tissue were found. The liver lesions were generally similar to those found in the heart, the large areas of necrosis also containing many foreign body giant cells surrounding clumps of amorphous basophilic material. In many of the rabbits injected with SEP there was also a marked increase in serum concentration of glutamic-oxalacetic transaminase (GOT), sorbitol dehydrogenase (SOD), and total serum lipids within 24 hours of the administration of SEP, indicating that tissue damage occurred soon after the injection.

The purpose of the present work is to study further the toxic effects of SEP in rabbits. Tissue damage induced in rabbits by group A streptococcal sonicates will also be described.

Materials and Methods. Bacterial strains. Group A streptococcus, type 6, was obtained from the Central Government Laboratories, Ministry of Health, Jerusalem. Streptococcus mitis was isolated from human saliva following growth on a mitis-salivarius medium (Difco). The streptococci were cultivated in brain heart infusion broth (Difco), harvested from the logarithmic phase of growth, washed in saline buffered with 0.025 *M* phosphate, pH 7.4, resuspended in buffer and kept at 4°C.

Streptococcal extracellular products (SEP). A lyophilized preparation of culture supernates obtained from a type 4 streptococcus grown in a chemostat was employed. The SEP contained at least 12 antigens as determined by immunoelectrophoretic analysis using rabbit antiserum. A detailed description of the method of preparation of SEP, its antigenic composition and enzyme content is given elsewhere (2).

Preparation of streptococcal sonicates. Washed streptococci obtained from 2 liters of culture were subjected to sonic oscillation in a 9 Kc Raytheon sonic oscillator for 90 min. Following the disruption, the bacterial debris was removed by centrifugation at 36,000*g* for 30 min at 4°C. The supernatant fluid was dialyzed overnight against 0.01 *M* phosphate buffer, pH 7.4, passed through a Millipore filter (0.22 μ) and kept at —25°C.

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Ion-exchange chromatography of streptococcal products. SEP. The SEP was examined on 10 × 1-cm columns of DEAE-Sephadex X-50 (Pharmacia) by stepwise salt elution techniques described previously (4). Briefly, SEP was dialyzed for 18 hours at 4°C against 0.05 *M* phosphate, pH 7.4. Twenty mg of the dialyzed SEP were applied to the column previously equilibrated with this buffer and the material not adsorbed to the column was collected (Fraction A). The bound antigens (Fraction B) were eluted with 1.0 *M* NaCl buffered with 0.01 *M* phosphate, pH 7.5. *Sonicates.* Sonicates obtained from group A streptococci were examined by chromatography on ECTEOLA-cellulose, using a linear salt gradient as described previously (4); amounts containing 200 mg of protein and 2 mg of rhamnose were applied to a 10 × 1.5-cm column. Following chromatography, the various fractions eluted were pooled, concentrated by pervaporation, dialyzed overnight against saline, and kept at —25°C.

Immunization of rabbits. Rabbits weighing 2–3 kg were immunized with whole SEP, or with the fractions obtained from SEP by ion-exchange chromatography, or with streptococcal sonicates. All materials were incorporated in Freund's complete adjuvant (Difco) for injection. Three weekly injections were given, followed 1 week later by an intramuscular injection of the same material without adjuvant. The animals were bled 10 days following the last injection.

Immunological techniques. Sera of both immunized and nonimmunized animals were analyzed for the presence of precipitating antibodies by immunoelectrophoresis and double-diffusion, according to established procedures, using rabbit antisera or pooled human γ-globulin.

Analytical methods. Prior to the experiment, and at various intervals following injection of the streptococcal products, the sera of the animals were analyzed for their content of GOT, by the method of Reitman and Frankel (5), and SOD, as described by King (6). Total lipids were determined by the method of Kunkel *et al.* (7). Protein was determined according to the method of Lowry *et al.* (8). Rhamnose was determined according to Dische and Shettles (9).

Histological techniques. The animals were sacrificed by intravenous injection of sodium pentothal, sections were obtained from all parts of the heart (base to apex), for fixation in 10% neutral formalin, as well as samples from liver, kidney, spleen, diaphragm, and tonsil. The tissues were embedded in paraffin and 5–6 μ sections were stained with hematoxylin and eosin.

Results. Pathological and enzymatic changes in rabbits injected with SEP, and some of its fractions, and with sonicates. SEP. Seven rabbits were injected with 2.5–4 mg of SEP and were sacrificed 7 days later. Table I shows that five of the rabbits had cardiac lesions and five had liver lesions. The morphology of

TABLE I. Pathologic Lesions, and Levels of Glutamic-Oxalacetic Transaminase, Sorbitol Dehydrogenase and Total Lipids, in Rabbits Injected with SEP.[a]

Rabbit no.	SEP injected (mg)	Lesions in		GOT (IU)		SOD (IU)		Serum lipids (mg/100 ml)	
		Heart	Liver	0	24 hours	0	24 hours	0	24 hours
1	2.5	2	—	10	x	4	x	490	1385
2	2.5	2, gr	—	10	65	5	11	657	850
3	2.5	—	nec	10	93	3	29	433	1168
4	4.0	2	gr	10	93	9	85	552	1260
5	4.0	3	gr	12	120	5	109	723	958
6	4.0	—	gr	11	x	5	x	506	1630
7	4.0	2	nec, gr	10	68	8	6	643	1595

[a] Abbrev.: 2 = necrosis with extensive inflammatory exudate; 3 = severe myocarditis; nec = coagulation necrosis; gr = granulomatous inflammation; and x = venous collapse, no blood drawn.

the lesions obtained in this group was essentially similar to those described previously (2). The cardiac lesions consisted of foci of myofibril degeneration infiltrated with granulocytes and monocytes. A typical giant cell granuloma containing many clumps of basophilic and amorphous material was found in one animal. In the liver of four rabbits, multiple foci of coagulation necrosis surrounded by many multinucleated giant cells were found. Of these seven rabbits, the GOT was elevated in four, the SOD in three and the serum lipids in all.

The data suggest that injury to the tissues probably occurred soon after the injection. To test this, three rabbits received 10 mg of SEP in the knee joint and two received 3 mg of SEP intravenously; three control animals were injected with 5 mg of SEP heated to 100°C. Enzyme determinations were performed prior to injection and 2, 4, 6, 8, and 24 hours following the injection.

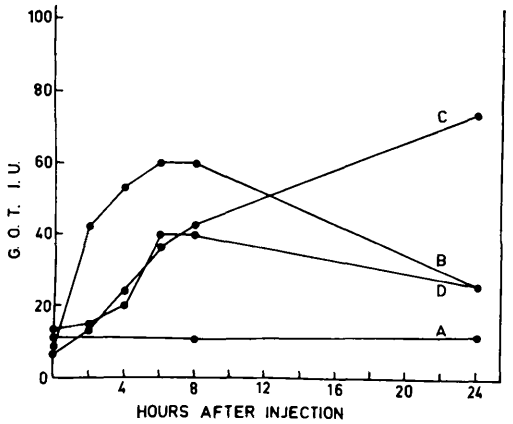

FIG. 1. Glutamic-oxalacetic transaminase levels in serum of rabbits injected with SEP: (A) SEP heated to 100°C, intratonsillar injection; (B) SEP 3 mg, intratonsillar injection; (C) SEP 5 mg, intra-articular injection; and (D) SEP 3 mg, intravenous injection. Each point represents the means of 3 animals.

Figure 1 shows that levels of GOT were elevated in all the rabbits injected with SEP 2–4 hours following the injection. The GOT levels reached a maximal value between 8 and 24 hours. All the animals injected with SEP, which were sacrificed 24 hours following injection, had many small areas of necrosis both in the heart and in the liver. The necrotic areas were infiltrated with polymorphonuclear leucocytes. On the other hand, no rise in GOT or pathological alterations were found in three rabbits injected with heat-inactivated SEP. For comparison with SEP, five rabbits were injected intratonsillarly with 25 mg of a preparation of streptokinase (SK) (100,000 units) and streptodornase (SD) (25,000 units) (Lederle Laboratories). This preparation, obtained from a group C streptococcus, contained, in addition to SK and SD, two esterases which split β-naphthylacetate and para-nitrophenylphosphate and showed the presence of four precipitating lines when tested by immunoelectrophoresis with rabbit antiserum to SEP. None of the animals injected with SK–SD had an increased level of GOT or SOD, or pathological lesions in any of the organs tested.

Injections of SEP fractions. Experiments were performed to isolate from SEP the active factor(s) responsible for tissue damage. By stepwise elution on DEAE-Sephadex, two fractions were obtained: fraction A, unadsorbed to the column in 0.05 *M* phosphate buffer, pH 7.4, and fraction B, adsorbed and then eluted from the column with 1.0 *M* NaCl. Fraction A was found to contain five antigens (Fig. 2) and to possess deoxyribonuclease (5,000 units/mg), hyaluronidase (20,000 units/mg), diphosphopyridine nucleotidase (1,600,000 units/mg) and streptolysin 0 (SLO) (100 units/mg). Fraction B contained six to seven antigens and was found to possess traces of the above mentioned enzymes, an

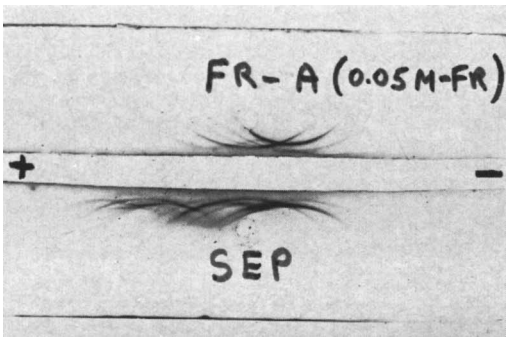

FIG. 2. Immunoelectrophoretic analysis of fraction A and of whole SEP, vs rabbit antiserum.

antigen adsorbed to mammalian cells (10), an acid phosphatase and an esterase.[3]

The two fractions were each injected intratonsillarly into four rabbits. Only fraction A caused a rise in GOT and SOD (Fig. 3) and

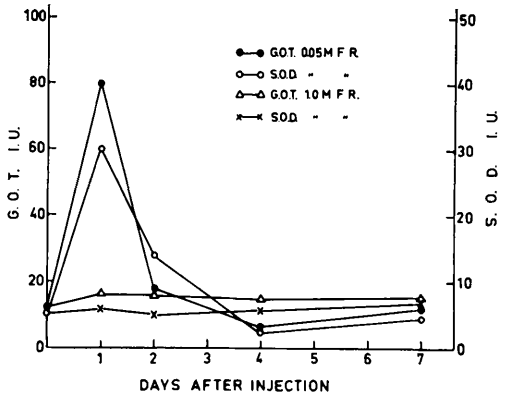

FIG. 3. Glutamic-oxalacetic transaminase and sorbitol dehydrogenase levels in rabbits injected with fraction A and fraction B of SEP. Each point is the mean value of 4 rabbits.

induced lesions. All of the animals injected with the 1.5 mg of fraction A had liver lesions and all developed heart lesions. All of the animals had elevated levels of GOT and three of the four had elevated SOD levels. Further work and the isolation and characterization of the active component(s) present in the fraction A is underway.

In another series of experiments six rabbits were injected with a mixture of SEP (2 mg) and sonicates containing 10 mg of protein. One of the rabbits developed severe myocarditis and three of the rabbits had typical liver lesions, but no lesions different from those described above were found.

Injection of sonicates. In preliminary experiments on disrupted streptococci we had failed to induce any pathological alterations in mice following intraperitoneal injection of 4.0 mg of cell wall material (58 mg of protein and 0.8 mg of rhamnose), as described by Schwab and Cromartie (11), despite the fact that these sonicates produced typical nodular lesions in the skin. Since rabbits were found to be susceptible to SEP, the effect of sonicates on rabbits was tested. Eleven rabbits were injected intravenously with streptococcal sonicates which contained 4 mg of protein and 100–500 μg of rhamnose. The sonicate preparations employed did not contain any traces of SLO, hyaluronidase, diphosphopyridine nucleotidase, or proteinase. Immunoelectrophoresis with rabbit antiserum showed the presence in the sonicates of at least six precipitating lines, most of which migrated to the anode (11). There was some cross-reaction between anti-SEP serum and the sonic extracts, but no common antigen between SEP and sonicates could be identified (12).

Prior to injection and 24 hours following injection, the levels of GOT and SOD were determined in all rabbits. The animals were sacrificed 7 days following the injection. Of the animals injected with 3 mg of sonicate, two had cardiac lesions and three had liver lesions. The GOT was slightly elevated in four animals and SOD in one animal. On the other hand, severe myocardial and hepatic lesions accompanied by an increase in GOT, SOD, and total lipids were found in five animals injected with large amounts of sonicate (Fig. 4). The pathological lesions in the

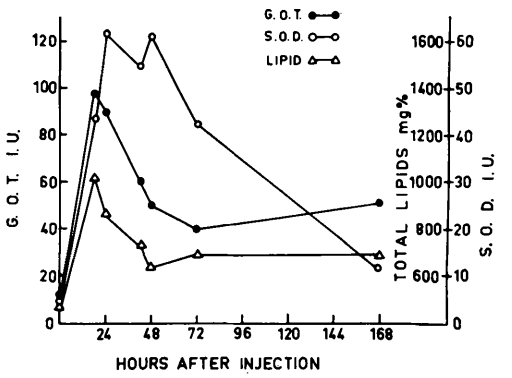

FIG. 4. Glutamic-oxalacetic transaminase, sorbitol dehydrogenase, and total serum lipid levels in animals injected intravenously with sonicate preparation containing 45 mg of protein and 500 μg of rhamnose. Means of 4 rabbits.

heart and the liver were essentially similar to those induced by SEP (2), or by sonicates derived from streptococcus mitis (12). The heart lesions consisted of foci of degeneration with granulomas (Fig. 5). The liver lesions consisted of multiple foci of liver degener-

[3] Ginsburg, I., to be published.

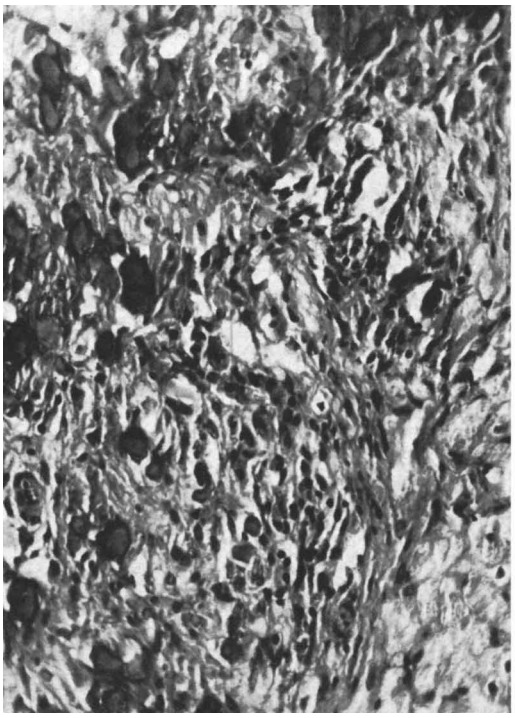

FIG. 5. Myocardial lesion in a rabbit 7 days following intratonsillar injection of group A streptococcal sonicate. ×270.

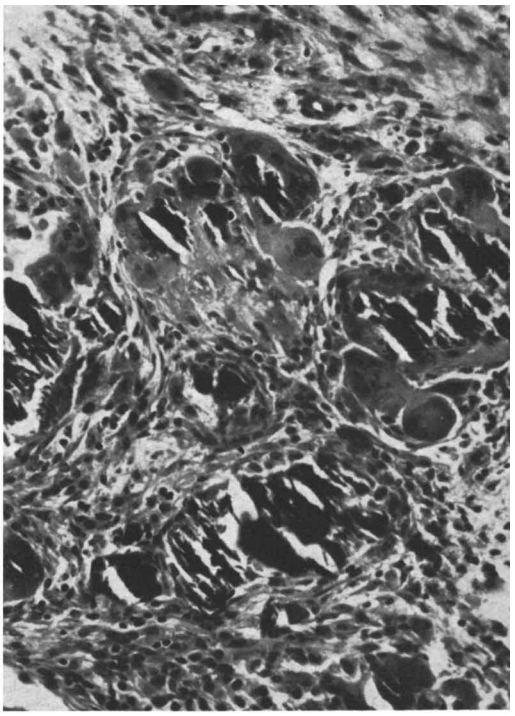

FIG. 6. Liver lesions in a rabbit 7 days following the intratonsillar injection of group A streptococcal sonicate. ×420.

ation infiltrated with inflammatory cells containing many multinucleated giant cells (Fig. 6).

Discussion. The data show that rabbits injected with a group of streptococcal antigens, not adsorbed to a positively charged adsorbant at pH 7.4, develop cardiac and liver lesions.

Since neither heated SEP nor a pool of negatively charged antigens from SEP nor a preparation of SK–SD caused tissue damage or a rise in serum enzymes, the role of the positively charged antigens seems to be specific. Fraction A was found to contain deoxyribonuclease, hyaluronidase, diphosphopyridine nucleotidase, and SK and to contain at least five antigens (Fig. 2). It remains to be seen whether the toxic agent(s) in fraction A are associated with any of the known streptococcal products or is due to a still unknown factor.

The appearance of tissue damage was usually associated with an increase in serum GOT, SOD, and serum lipids, which could be detected as early as 2 hours after the injection of SEP or fraction A. Thus, an increase in the levels of these enzymes may be useful for the early diagnosis of tissue damage caused by streptococcal products (2).

Toxic effects in rabbits similar to those induced by SEP, and accompanied by a rise in GOT, SOD, and total lipids, were also observed following administration of group A sonicates. The similarity in the toxic effects of SEP and sonicates may be due to the presence of a common toxic component in both preparations or to the presence of different factors similarly affecting the heart and liver. Since the sonicate preparation employed did not contain any traces of deoxyribonuclease, hyaluronidase, SK, diphosphopyridine nucleotidase, or SLO, it would appear that the toxic factor in the sonicate is not associated with any of these known streptococcal products.

Further studies on the isolation and characterization of toxic components present in SEP and the sonicates are in progress.

Summary. Rabbits injected with streptococcal extracellular protein (SEP) developed degenerative and infiltrative lesions in the heart and liver, with coagulation necrosis and multinucleated giant cells in the latter organ. The majority of these rabbits also showed elevated levels of glutamic-oxalacetic transaminase, or of sorbitol dehydrogenase, and all showed elevated serum lipids. These biochemical indications of cell injury could be found within 2 to 4 hours after a first injection of SEP. No such biochemical or pathologic effects were found following an injection of heated SEP or a commercially available streptokinase–streptodornase preparation from group C streptococcal culture. In a preliminary fractionation of SEP all the activity was found in a fraction not adsorbed to DEAE cellulose at pH 7.4 (0.05 *M* PO_4). The active fraction contained at least five antigens and four of the known streptococcal enzymes. Injection of extracts of sonically disrupted streptococci produced similar biochemical and pathologic changes. There was some cross-reacting material between the sonicates and anti-SEP serum.

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Hyperphagia in the Insulin-Treated Rat* (32909)

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We have described recently certain metabolic effects of hyperphagia in the hypothalamic-hyperphagic rat (1). In the early dynamic phase of hyperphagia, increased body weight gain appeared to be a direct effect of increased energy intake not balanced by the small increase of spontaneous activity; increased lipogenesis and decreased lipolysis *in vitro* were observed with no evidence of altered thyroid activity. In the later static phase of hyperphagia, spontaneous activity was not increased, metabolic patterns were altered in the direction of increased lipogenesis and decreased lipolysis, and there appeared to be thyroid hypofunction. In 1940, MacKay *et al.* (2) demonstrated that protamine zinc insulin (PZI) increases food intake and body weight gain. This observation has been amply confirmed by several groups of investigators and the potent property of PZI is apparent from the observation of Beaton *et al.* (3) that PZI overcomes the

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