Dialyzable Form of an Extracellular Streptococcal Toxin Causing Histopathologic and Biochemical Changes in Rabbits[1] (36604)

EPHRAIM GAZIT,[2] ISAAC GINSBURG, AND T. N. HARRIS

The Children's Hospital of Philadelphia, Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19146; and the Laboratory for Microbiology and Immunology, Faculty of Dental Medicine, Alpha Omega Research and Postgraduate Center, The Hebrew University, Jerusalem

Group A streptococci are known to produce a wide variety of toxic compounds (1–8). The biological activities of some of these toxins have been investigated, and among the effects found were histologic changes in tissues, alterations in levels of serum enzymes, and pyrogenicity (4–6).

Tissue changes classified as necrotic, granulomatous or infiltrative have been described, with localization in the heart, liver, skin or diaphragm (5, 7). Elevation of serum enzymes such as glutamic-oxalacetic transaminase (GOT), increase in serum lipids and fever were other effects of the injected toxins (8). It has been reported from these laboratories that extracellular products derived from Group A, type 4, streptococci grown in steady-state culture, induced severe tissue damage on intravenous injection (5). The toxic material was in the Sephadex G-150 excluded fraction of an ammonium sulfate precipitate of the supernate of the streptococcal culture. The present report describes studies undertaken in an attempt to separate further a causative agent.

Materials and Methods. Streptococci of group A, type 4, strain H 44 were grown in steady-state culture, (9) using a completely synthetic medium (Table I) with separate pH control (10).

Culture supernatants were collected by centrifugation and concentrated tenfold by pervaporation. The concentrate was treated with an equal volume of ice-cold 0.5 *M* trichloraocetic acid, with constant stirring, for 2 hr in the cold. The mixture was centrifuged in the cold in a Sorvall SS_1 centrifuge at 16,000 rpm for 30 min. The resulting supernatant was treated with ice cold 95% ethanol for 18 hr, and the sediment thus produced was dissolved in pyrogen-free distilled water. The alcohol precipitation was repeated, and the readily soluble alcohol precipitate was examined on a Sephadex column. The effluent was monitored by OD_{280}, and absorbing fractions were lyophilized and stored at $-20°$ until used.

Random bred albino rabbits of 2–3 kg were obtained from a local supplier. For testing the biological activity, rabbits were bled from the lateral ear vein before the intravenous injection and 24 and 48 hr thereafter. Serum GOT was determined by the use of Sigma Kit, Cat. No. 505 (Sigma Chemical Corp., St. Louis, MO 63118), and total lipids as described by Kunkel, Ahrens and Eisenmenger (11). For histological examination, the heart, liver, diaphragm and kidneys were removed from animals which had been given a lethal dose of Nembutal. The tissues were fixed in neutral formalin, and embedded in paraffin. Microscopic sections were stained with hematoxylin and eosin.

Results. In the preliminary experiments aliquots were examined by Sephadex G-150 and G-50. Both the excluded and nonexcluded fractions contained materials which were lethal to rabbits upon intravenous injection in adequate amounts, or which caused elevated levels of serum lipids and GOT if smaller doses were injected. Of the fraction

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[2] Present address: Department of Pediatrics, Tel Hashomer Hospital, Tel Hashomer, Israel.

TABLE I. Composition of Synthetic Medium for Steady-State Cultivation of the Streptococci (moles/liter).

Amino acids:			
DL-Alanine	3.8×10^{-3}	L-Lysine	1.1×10^{-3}
L-Arginine HCl	3.6×10^{-3}	DL-Methionine	0.56×10^{-3}
Asparagine (anh)	9.3×10^{-3}	DL-Phenylalanine	0.94×10^{-3}
L-Cysteine HCl	3.4×10^{-3}	L-Proline	0.25×10^{-3}
L-Glutamic acid HCl	9.4×10^{-3}	DL-Serine	22.6×10^{-3}
Glycine	1.5×10^{-3}	DL-Threonine	3.8×10^{-3}
L-Histidine HCl	2.7×10^{-3}	DL-Tryptophan	0.22×10^{-3}
DL-Isoleucine	1.8×10^{-3}	L-Tyrosine	0.49×10^{-3}
DL-Leucine	2.8×10^{-3}	DL-Valine	5.6×10^{-3}
Other constituents:			
K_2HPO_4	0.0500	Uracil	9.0×10^{-5}
Na_2HPO_4	0.0500	Adenine SO_4	4.3×10^{-5}
Thioglycolic acid	0.0088	Glucose (reagent)	0.0500
$CuSO_4 \cdot 5H_2O$	4.0×10^{-6}	$CaCl_2 \cdot 2H_2O$	5.00×10^{-5}
$ZnSO_4 \cdot 7H_2O$	2.8×10^{-6}	Niacin	1.05×10^{-5}
$FeSO_4 \cdot 7H_2O$	3.0×10^{-6}	Pyridoxine HCl	7.7×10^{-6}
$MnCl_2 \cdot 4H_2O$	2.0×10^{-6}	Ca Pantothenate	2.10×10^{-5}
Riboflavin	6.7×10^{-6}	Thiamin HCl	3.85×10^{-6}
$MgSO_4$	4.2×10^{-3}	Riboflavin	1.59×10^{-6}
$NaHCO_3$	0.025	Biotin	4×10^{-10}

not excluded by Sephadex G-50 continued examination on Sephadex G-25 and G-15

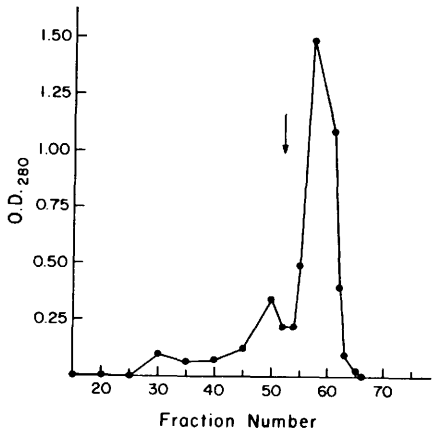

FIG. 1. Analysis on Sephadex G-25 of the TCA soluble, ethanol precipitated, fraction of the streptococcal culture supernate concentrate. OD_{280} of successive fractions from the column. The limit of the excluded volume is shown by the vertical arrow. Fractions from 27 to 62 pooled in groups of 7 for injection into groups of 3 rabbits. The rabbits injected with fractions 55–62 showed a mean serum GOT value of 124, 24 hr later. All other groups, 27–34, 35–41, etc., yielded mean GOT levels between 11 and 15.

yielded a toxic fraction which was not excluded by G-25 (Fig. 1) and only partially excluded by G-15 (Fig. 2). To determine the approximate molecular weight vitamin B_{12} (mol wt 1357) was similarly examined; the toxic material was eluted almost at the same place (Fig. 2). Other properties were examined in the light of the low molecular weight. The toxic material was found to be dialyzable and heat resistant. Boiling for 2 min did not reduce its potency to cause either death or the elevation of enzyme levels.

As another approach to an estimate of molecular size of the active material, preparations were examined by ultracentrifugation. Data from ultracentrifugal analysis of a typical toxic preparation are shown in Fig. 3, the exposures being at zero time and at 16-min intervals thereafter, from a run at 59,800 rpm in a Spinco model E analytic ultracentrifuge. All of the material appears to be sedimenting in one peak, the $S_{20,w}$ of which was calculated to be 0.3.

Autoclaved medium, in which bacteria had not been grown, was treated in the same way as the culture supernate. Material absorbing at OD_{280} and not excluded by Sephadex

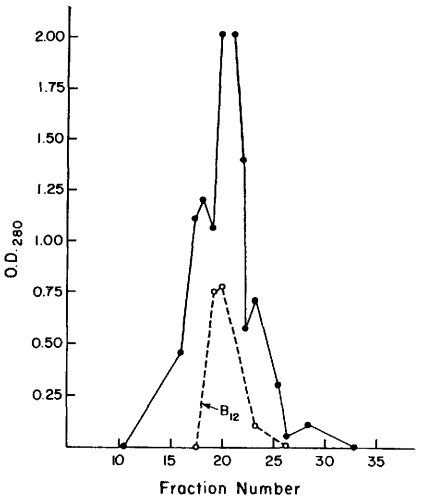

FIG. 2. Analysis of same material on Sephadex G-15, with vitamin B_{12} on same column as a marker. Fractions from 9 to 32 pooled in groups of 8 for injection into groups of 3 rabbits. The rabbits injected with fractions 17-24 showed a mean serum GOT value of 139, 24 hr later. The other pools, 9–16 and 25–32, yielded mean GOT levels of 17 and 19.

G 25 was found. This resembled in its elution pattern the culture supernatant material (Fig. 4). Rabbits were injected intravenously with this fraction in amounts up to 10–50× the amounts of culture supernate which had produced the toxic effects, but there was no lethal effect or elevation of serum GOT. This medium component has, however, made it impossible, thus far, to purify the toxin completely and thus study its chemical structure or even the absolute concentration required for the biologic effects. Of the low molecular weight fraction of culture supernates, amounts in the range of 2.5–5.0 mg/kg were found to be lethal to rabbits, but since the ratio of toxin to components of the medium present in this fraction was not known, the LD_{50} could not be ascertained.

Intravenous injection of this fraction caused severe damage to the myocardium (Fig. 5) and the liver (Fig. 6). The histopathological picture resembled those described earlier in these studies (5, 8) with the exception that calcification of the necrotic areas was rarely seen.

Discussion. In previous reports from these laboratories, Sephadex G-150 excluded material from ammonium sulfate precipitates of supernates from streptococcal group A type 4 cultures caused tissue damage when injected into rabbits (5). In the present study the ammonium sulfate precipitation was replaced by trichloroacetic acid, which caused denaturation of most of the high molecular weight protein, resulting in the liberation of material of low molecular weight. Precipitation with ethanol further purified the preparation.

The tissue changes caused by these substances were quite similar to those caused by the G-150 excluded material in our earlier studies (5, 8). It is possible that the trichloroacetic acid treatment and the alcohol precipitation cleaved a large molecule, yielding a small fraction which retained its toxicity. On the other hand, it may be that the toxic material, as originally produced in the culture, is a small molecule which can be bound to protein, and which in its native form escaped notice in the earlier studies because of its dialyzability. Recently Kim and Watson described another preparation from streptococcal culture supernates which is toxic to rabbits (4). Our preparation is different from

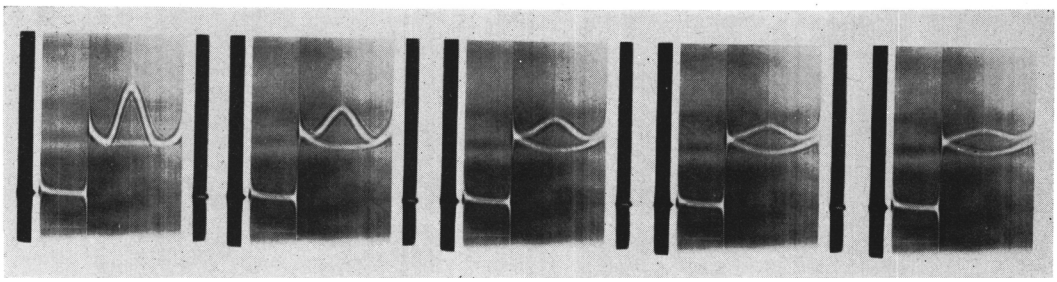

FIG. 3. Ultracentrifugal analysis of streptococcal toxic fraction, at 59,800 rpm. Exposures at intervals of 16 min.

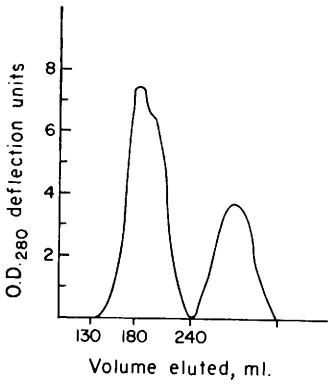

FIG. 4. Concentrate of unseeded medium on Sephadex G-25, column 2.5 × 45 cm. Volume eluted vs OD deflection on Texas Instrument Co. Recti-Riter (Gilson fraction collector and UV monitor).

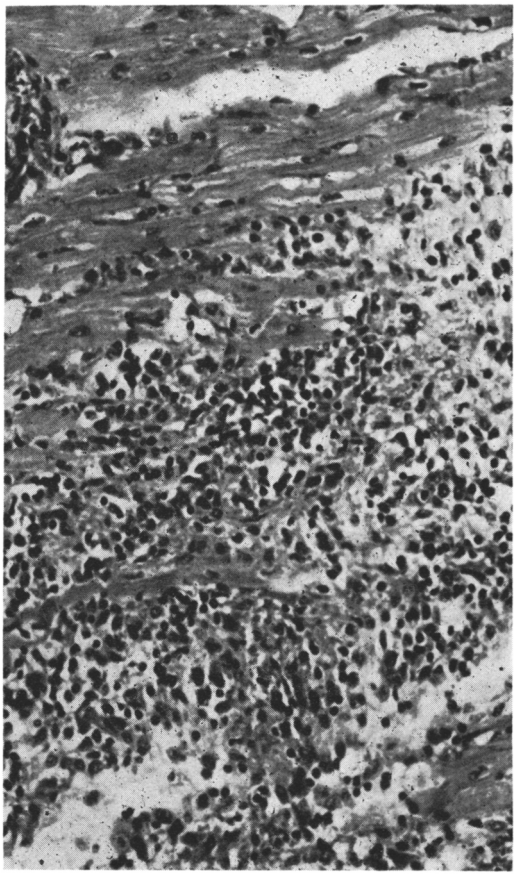

FIG. 5. Toxic lesion, largely infiltrative, in cardiac muscle of rabbit 2 days after injection of 1 mg of the dialyzable streptococcal toxin. ×125.

theirs in its lower molecular weight and its heat stability.

Thus far, our studies were conducted only with group A streptococci of type 4. It would be interesting to find whether other groups and strains of streptococci produce a similar toxin.

Such a potent toxin again draws attention to the pathogenicity of group A streptococci.

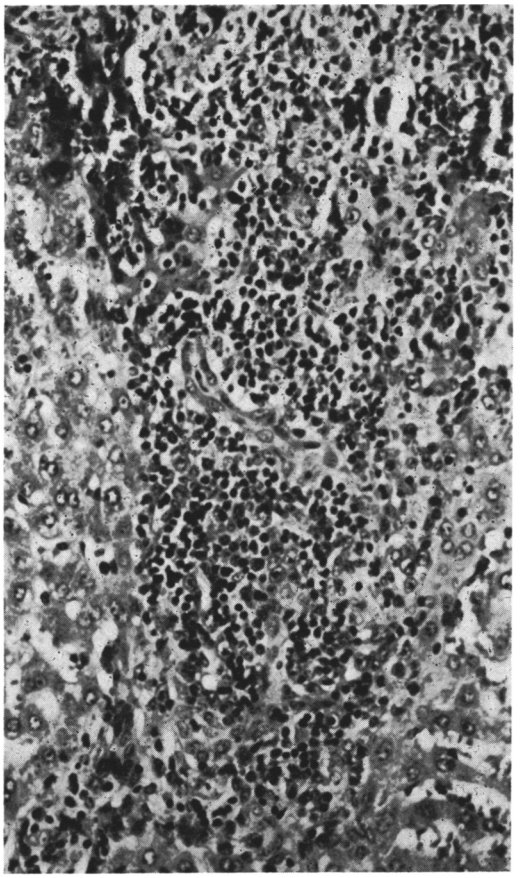

FIG. 6. Same as Fig. 5; liver; ×125.

Whether it acts similarly in the human being is not known, but preliminary experiments with primates have shown an elevation of serum GOT after an intravenous injection of this toxin (unpublished data).

Summary. Earlier reports had described the presence, in supernates of streptococcal steady-state cultures, of a macromolecular toxin which causes infiltrative and necrotic lesions in the heart and liver of rabbits and

changes in the serum level of certain enzymes and lipids. In the present study, following treatment of the culture supernate concentrates with trichloroacetic acid and ethanol, and dialysis, material with these biologic activities has been found, in the dialysate, and was not excluded by Sephadex G-15. The molecular weight of the toxin, by this criterion, is near that of vitamin B_{12}.

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