IDENTIFICATION OF A TOXIC CELLULAR COMPONENT OF GROUP A STREPTOCOCCI AS A COMPLEX OF GROUP-SPECIFIC C POLYSACCHARIDE AND A PROTEIN*

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Chronic multinodular skin lesions were produced in rabbits injected intradermally at a single site with a sterile extract of sonically disrupted Group A streptococcal cells (1). These lesions, which consisted of separate nodules scattered over areas as large as 12 cm. in diameter, presented several remarkable features. The injury to dermal connective tissue was evident within 48 hours after injection, suggesting that the material had a primary toxic effect. The reaction was observed to continue for as long as 30 days in the first series of experiments (1), and subsequent studies have revealed it to continue for at least 60 days after a single injection.

In the gross, the process was characterized by periods of abatement followed by either exacerbation of the subsiding lesions or the development of additional nodules in areas where the lesions have previously completely subsided. The initial nodules and the recurrent nodular lesions were associated with focal necrosis of dermal connective tissue. What appeared to be acute focal necrosis was noted during the first 48 hours after injection and as late as 60 days after the animals were inoculated. As the process evolved, mononuclear and giant cells were found in the tissues along with the areas of acute focal necrosis.

The gross and microscopic changes suggested that a single injection of this sterile extract could initiate a mechanism of injury that caused recurrent acute focal damage of connective tissue extending over a period of at least 60 days. Comparative study of extracts of various species of streptococci suggested that this toxic material was a cellular component characteristic of Group A streptococci (2). It is the purpose of this report to record data associating the toxic activity with a complex macromolecule containing the Group-specific C polysaccharide.

Materials and Methods

Extraction of Cells.—The cultivation, harvesting, and extraction of the organisms has been described (1). The cells from an 18 hour broth culture were washed 2 times with cold saline

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and resuspended in pH 7.0 phosphate buffer of $\Gamma/2$ 0.1. This suspension was then subjected to sonic oscillation for 1 hour in a Raytheon 9 kc. sonic oscillator, centrifuged at 36,000 *G* for 30 minutes in a Spinco No. 40 rotor, and the supernatant filtered through a Selas 02 filter to yield the sterile crude extract. A Type 1, Group A streptococcus was used in these studies.

Electrophoresis.—Electrophoretic separation was carried out using counter-current electrophoresis in a Spinco model H electrophoresis apparatus. The separations were done in pH 7.65, $\Gamma/2$ 0.1 phosphate buffer.

Chemical Analyses.—Nitrogen was determined by the micro-Kjeldahl method utilizing Nessler's reagent. Protein was determined by ultraviolet absorption at 215 and 225 mμ in a Beckman DU spectrophotometer as described by Waddell (3), and also by the biuret method, using crystalline bovine albumin as a standard. Rhamnose was determined by the method of Dische and Shettles (4). Glucosamine was determined by the method of Johnston *et al.* (5). The method of McRary and Slattery (6) was used to determine pentose. It was necessary to correct the pentose readings for the amount of methylpentose present since the concentration of rhamnose was relatively high in the preparations under study. Deoxyribose was estimated as described by Burton (7). Total reducing sugars were determined by the method of Somogyi as modified by Nelson (8). Sugar was also determined with the anthrone reagent as described by Seifter and Dayton (9). Phosphorous was measured by the method of Fiske and SubbaRow (10). Lipide phosphorous determinations were made on dried samples extracted 3 times at room temperature with 3:1 alcohol-ether. Paper chromatography was carried out on samples hydrolyzed with 4 N HCl in sealed ampules for 4 hours in a boiling water bath and evaporated to dryness. An ascending technique using butanol–acetic acid–water solvent and a descending method using the ethyl acetate–acetic acid–water solvent described by Jermyn and Isherwood (11) were employed. Aniline phthalate and *p*-phenylene-diamine were used to identify the spots.

The centrifuge fractions are designated by the terminology suggested by Alexander (12). The letter p or s refers to precipitate or supernate, the prefix indicates the force in 1,000 *G* and the suffix refers to time in minutes. The number following the hyphen refers to the number of centrifugations and resuspensions under these conditions.

RESULTS

The initial problem in fractionation was quantitating the lesion-producing activity. It was observed that the area of the lesion and the time required for gross appearance were proportional to the relative concentration of the crude extract. The area of involvement, in millimeters, divided by time of appearance, in days, is an index of the activity. Fig. 1 shows that the log of the index is a function of the relative concentration. Thus, the index obtained from the average of 5 to 6 rabbits is used as a means of quantitating the activity. A similar proportionality has been demonstrated with the purified fractions.

Differential centrifugation of the extract was carried out, and the activities of fractions obtained between 10,000 and 140,000 *G* were compared. In this experiment the suspension of sonic disrupted cells was first centrifuged at 2750 *G* for 30 minutes and the supernatant filtered through a Selas 02 filter. As can be seen in Table I, the greatest part of the activity, based on weight of nitrogen injected, was observed in the fraction collected between 36 and 75,000 *G*. Therefore, on the basis of these studies the first procedure in purification consists of 2 successive centrifugations of the crude extract at 36,000 *G* for 30 minutes each after which a precipitate is collected at 75,000 *G* and washed 3 times with pH 7, $\Gamma/2 = 0.1$, phosphate buffer.

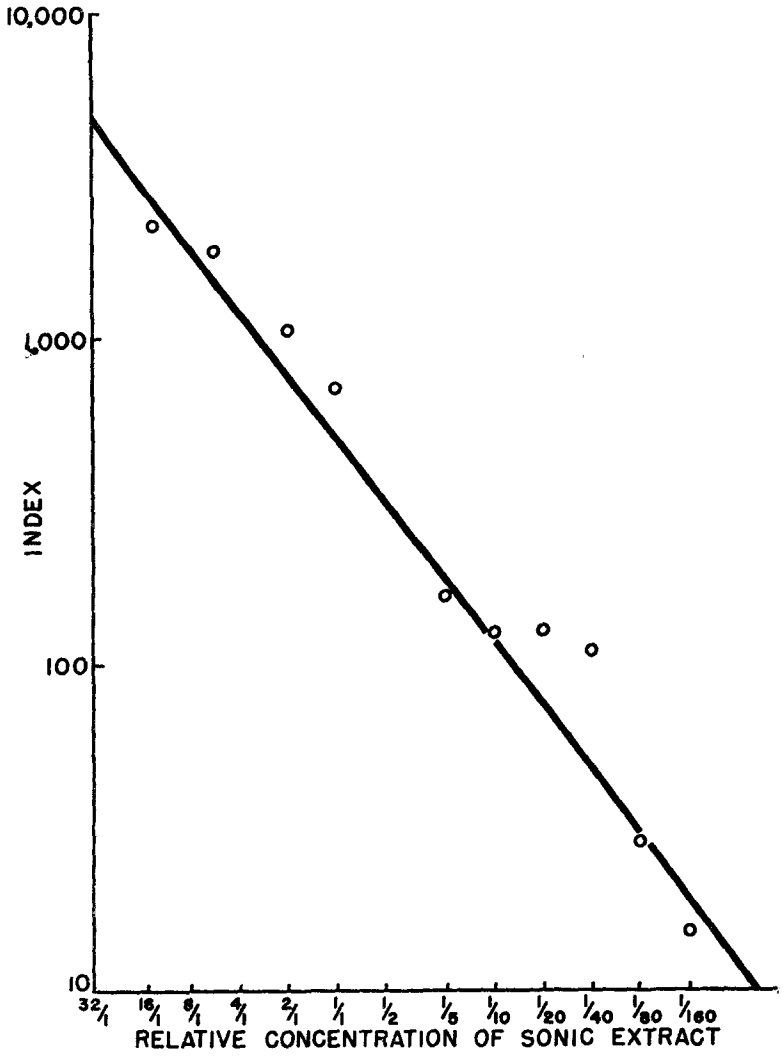

FIG. 1. Relationship of log of index $\left(\frac{\text{area of lesion in millimeters}}{\text{time of appearance in days}}\right)$ to relative concentration of sonic extract.

The particulate nature of the active substance was investigated further by resuspending the 36p30-2 and the 75p60-2 precipitates in pH 7 phosphate buffer and subjecting each suspension to additional sonic vibration for 1 hour. The 36p30 suspension was then centrifuged at 36,000 *G* for 30 minutes and the sediment resuspended to the original volume. This sediment, the supernate, and an aliquot of the initial suspension were then injected intradermally into rabbits. In a similar manner the revibrated 75p60 fraction was centrifuged at 75,000 *G* for 1

hour and the toxic activity of the resuspended precipitate, the supernate, and the whole suspension were compared.

The results are recorded in Table II. It is apparent that the activity associated with the large particle fraction can be further solubilized by additional sonic vibration. On the other hand, the activity associated with the 75p60

TABLE I

*Relative Activity of Ultracentrifuge Fractions of Sonic Extract**

Fraction	Index	N injected	$\frac{\text{Index}}{\mu g.\ N}$
		μg.	
10p30-1	731	60	12
25p30-1	255	30	8
36p30-1	384	21	18
75p60-1	641	19	34
96p60-1	263	60	4
140p120-1	12	50	0.24
140s120-1	13	150	0.08

* The suspension was first centrifuged at 2750 × *G* for 30 minutes and the supernatant filtered through a Selas 02 filter.

TABLE II

Effect of Sonic Vibration on Sedimentation Properties of Ultracentrifuge Fractions

Sample	Index	μg./ml.	
		N	Rhamnose
36p30 (not revibrated)	614	1459	—
36p30 (revibrated, recentrifuged, sediment)	350	279	—
36p30 (revibrated, recentrifuged, supernate)	629	410	—
75p60 (revibrated 10 min.)	708	400	450
75p60 (revibrated 1 hr.)	662	400	450
75p60 (revibrated, recentrifuged, sediment)	606	158	350
75p60 (revibrated, recentrifuged, supernate)	67	156	75

fraction was only slightly influenced by additional sonic energy, suggesting that these particles represented a minimal macromolecular unit. It may also be noted that the toxic activity appears to be correlated with the concentration of rhamnose.

Further purification of the ultracentrifuge fraction 75p60 can be achieved by $(NH_4)_2SO_4$ fractionation; the activity being found in the supernate from 50 per cent saturation. The most satisfactory preparation of the active material, however, has been obtained by electrophoretic separation in a Tiselius cell of the components in the fraction obtained by differential centrifugation.

Fig. 2 shows the electrophoretic resolution of the ultracentrifuge fraction 75p60. It is important to mention that essentially the same pattern was obtained using veronal buffer pH 8.6. In Table III are seen the mobilities and the percentage of the total area of each of the 3 major components. This table also shows that the relative concentration of fraction 3, containing the active component, was increased by washing the precipitate 5 times rather than twice. Further washing had little effect on the relative composition, and the sediment became increasingly difficult to resuspend.

The trailing component, number 3, was removed from the descending side with a needle and represents a reasonably homogeneous fraction, although this cannot be accepted as an absolute criterion of purity. Components 1 and 2 were removed together as the leading com-

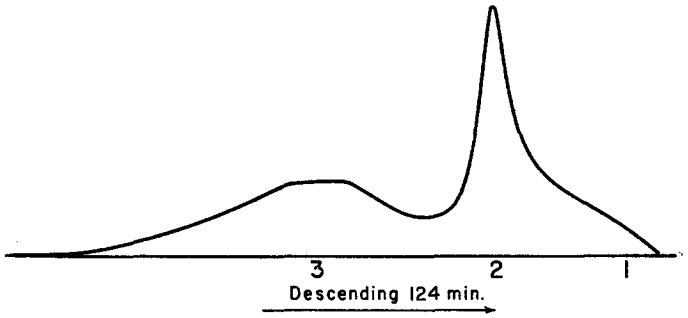

FIG. 2. Electrophoretic pattern of 75p60-4 fraction of sonic extract. Phosphate buffer, pH = 7.65, $\Gamma/2$ = 0.1, 6.06 v./cm.

ponent from the ascending arm. The activity of these fractions was determined by intradermal rabbit injection and in 4 experiments it was demonstrated that the trailing fraction had 30 to 40 times more lesion-producing activity than the leading fraction. These results are summarized in Table IV. The lesions produced with the trailing fraction have all the features, including remissions and exacerbations, observed with the crude extract.

A titration of the trailing component revealed the smallest dose giving a detectable lesion was 2.2 μg. A relapse was observed with 9.0 μg.

On the basis of these results a larger quantity of the trailing and leading components was collected from 40 L of cells, employing counter-current electrophoresis in several runs in the Tiselius cell. These fractions were then dialyzed in the cold against several changes of distilled water until the phosphorus and nitrogen values were constant. The analysis of these samples is summarized in Table V. The most impressive chemical difference between the active trailing fraction and the inactive leading component was found in the concentration of carbohydrate. The ratio of protein to carbohydrate was approximately 1 to 1 in the trailing fraction compared to a ratio of 10 to 1 in the leading component. The molar ratio of rhamnose to hexosamine in the

trailing fraction was 1.4, which is comparable to that reported by Barkulis (13) for the C substance in isolated cell walls. Rhamnose and glucosamine gave 59

TABLE III

Electrophoresis of 75p60 Fraction of Sonic Extract

Fraction	Mobility*	Per cent composition	
	($\times 10^{-5}$ cm.^{2}v^{-1}sec.$^{-1}$)	75p60-2	75p60-5
1	−3.11	17.5	12.0
2	−2.86	36.0	31.5
3	−2.00	47.0	57.5

* Average of 5 runs; PO_4 buffer, pH = 7.65, $\Gamma/2$ = 0.1, 6.06 v./cm.

TABLE IV

Relative Activity Index of Sonic Extract and Fractions

Sample	Index	N injected	$\frac{\text{Index}}{\mu\text{g. N}}$
		μg.	
Sonic extract (36s30-1)	1088.0	580.0	1.9
75p60-2	351.0	11.2	31.3
Trailing component	681.0	7.6	82.5
Leading component	16.0	7.6	2.1

TABLE V

Composition of Trailing and Leading Electrophoretic Fractions

Component	Per cent of dry weight	
	Trailing fraction	Leading fraction
Nitrogen	8.1	10.2
Phosphorous	1.1	0.75
Total sugar (as glucose)	23.0	6.0
Pentose	0	0.75
Rhamnose	24.0	2.0
Glucosamine	17.5	2.5
Remaining sugar (as glucose)	0	2.9
Protein	38.0	59.5

and 76 per cent respectively of the color reaction obtained with glucose in the Somogyi method of sugar determination. Thus, it may be seen from Table V that these two sugars accounted for all the carbohydrate present in the trailing fraction. In the leading fraction, sugar other than ribose, rhamnose, and glucosamine amounted to 2.9 per cent (as glucose) of the dry weight.

As a control on the specificity of each of the techniques used for quantitating these sugars, spectral absorption curves were obtained for the samples and a variety of known sugars. In the case of glucosamine and rhamnose there was nearly complete correspondence of the sample and the known sugar being estimated. Chromatographic analysis of the sugars revealed the presence of glucosamine and rhamnose in the trailing fraction; while in the leading fraction only galactose could be observed.

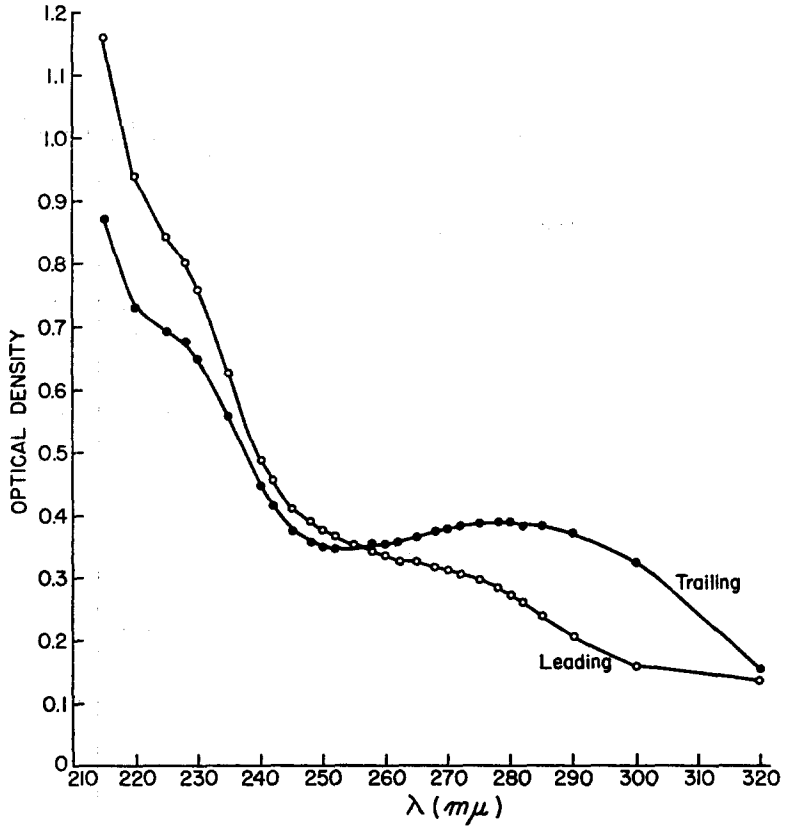

FIG. 3. Absorption curve of HCl extracts of trailing and leading fractions. Abscissa: optical density; ordinate: wave length in millimicrons.

No deoxyribose was detectable in 1.5 mg. of either fraction. Only a trace (0.03 per cent) of lipophosphorous was observed in the fractions. No evidence for the presence of other lipides was obtained employing the micro technique of Swahn (14), using either Sudan black B or osmic acid. The ultraviolet absorption spectrum of HCl extracts of the samples is shown in Fig. 3. The samples were hydrolyzed in 4 N HCl 4 hours in a boiling water bath. There is no evidence here of nucleic acid components in the trailing fraction. Absorption spectra of extracts prepared with 0.4 N $HClO_4$ for 15 minutes at 70°C. likewise displayed no evidence of nucleic acids.

From these results, 81 per cent of the dry weight of the trailing fraction and 71 per cent of the leading fraction can be accounted for. These yields are not inconsistent with results noted by others (15) working with comparable polysaccharide complexes from bacterial cells. The failure to account for 100 per cent of the sample is generally ascribed to bound water and the inaccuracy of the analytical methods.

TABLE VI

*Effect of Various Enzymes on Toxin**

Sample	Conditions of treatment	Decrease of N	N injected	Index	Index / N injected
		per cent	*μg.*		
Chymotrypsin treated toxin	Borate buffer, pH 8.5, $\Gamma/2 = 0.096$	40	62	368	5.9
			31	232	7.5
Trypsin treated toxin	Borate buffer, pH 8.5, $\Gamma/2 = 0.096$	35	68	410	6.0
			34	109	3.2
Toxin control	Borate buffer, pH 8.5, $\Gamma/2 = 0.096$	3	100	268	2.7
			50	130	2.6
Ribonuclease treated toxin	Phosphate buffer, pH 7.6, $\Gamma/2 = 0.10$	8	84	410	4.9
			42	164	3.9
Papain treated toxin	Phosphate buffer, pH 7.1, $\Gamma/2 = 0.10$	48	53	998	18.8
			27	492	18.2
Toxin control	Phosphate buffer, pH 7.1, $\Gamma/2 = 0.10$	—	103	744	7.2
			52	116	2.2
Pepsin treated toxin	HCl-KCl buffer, pH 2.2, $\Gamma/2 = 0.10$	37	64	173	2.7
			32	124	3.9
Toxin control	HCl-KCl buffer, pH 2.2, $\Gamma/2 = 0.10$	—	103	242	2.3
			52	154	2.9

* A 75p60-4 ultracentrifuge fraction was the toxin preparation used.

The presence of rhamnose and hexosamine indicates that the polysaccharide is the group-specific C substance of the cell. This was confirmed by a positive ring precipitin test which the trailing fraction gave with Group A specific antiserum. The leading component gave a ± reaction with the group-specific antiserum relative to a 2+ reaction shown by the trailing fraction.

Electron microscope studies revealed that activity was associated with discrete particulate material in all stages of fractionation.

From the fact that the trailing fraction appeared to be reasonably homogeneous during ultracentrifugation and electrophoresis, it seems that this analysis represents a macromolecular complex of polysaccharide and protein. To further elucidate the nature of the active moiety, aliquots of the active fraction were treated with crystalline ribonuclease and trypsin for 2 hours at 27°C. in 0.1 per cent concentrations. There was no significant difference in toxic activity between the control and the material treated with either enzyme. These studies were extended by utilizing several enzymes and carefully following the enzyme action to completion. The enzymes and the conditions under which they were used may be seen in Table VI. Each of the enzymes in a concentration of 0.005 per cent was added to an aliquot of a 75p60-4 fraction. Controls consisted of each enzyme without substrate, and aliquots of the 75p60 fraction subjected to the experimental conditions of each enzyme treatment. Following incubation for 1 hour at 30°C., samples were removed and the non-protein nitrogen determined. The pH was checked and a second portion of enzyme added. This was repeated until the increase in non-protein nitrogen became stationary. The per cent of protein digested by each enzyme under these conditions is shown in Table VI. The enzyme treated samples and their respective controls were then dialyzed against distilled water in the cold for 72 hours. The pH of the samples was adjusted to near neutrality and each was injected intradermally into 6 rabbits.

A preliminary titration of the 75p60 fraction used in this experiment was performed in order to determine a toxic concentration which was not in excess of that required to produce a typical lesion. The samples were also injected in a ½ dilution as well as undiluted. This was done because the relatively crude method of quantitating the lesion could conceivably fail to demonstrate an effect of proteolytic digestion which was less than complete. However, as seen in Table VI, the effect of enzyme treatment, especially with papain, was to increase the biological activity of the toxin. These results indicate that the polysaccharide moiety of the macromolecule is an essential part of the toxic material.

DISCUSSION

The component in the sonic extract of Group A streptococcal cells, responsible for the lesion under study, has been isolated by physical methods, involving ultracentrifugation and electrophoresis, and exhibited properties of a homogeneous preparation. This material was a polysaccharide-protein complex, the polysaccharide moiety having the chemical and serological characteristics of the group-specific C substance. From the studies employing a variety of enzymes and from consideration of the relative composition of an active and an inactive fraction, it appears that the group-specific polysaccharide plays an essential role in producing the toxic effect observed. This concept was supported by the relative solubility of the active material in $(NH_4)_2SO_4$ solutions, the relative heat stability, and the fact that this toxic activity is characteristic of Group A streptococci (1, 2).

Since Lancefield (16) established a sound basis for classification of streptococci, various investigators (17, 18) have observed that the group-specific

polysaccharide of Group A streptococci is chemically combined with a protein; but there have been no reports on the biological activity of the complex, other than antigenicity. Rather, most studies are concerned with the isolation of each entity. A serologically active form of the C polysaccharide has been isolated by Schmidt (19) and by Zittle and Harris (20). The molecular weight of this material was estimated by both groups to be about 8,000. When injected intravenously into mice it was excreted rapidly and no pathological changes were observed (19). We have observed that formamide extract preparations of C substance, comparable to the material Zittle (20) studied, produced in the gross no observable lesions following intradermal injection into rabbits. It would appear that if the C polysaccharide is involved in the development of the lesions described in this report, the physical state or chemical combinations of the polysaccharide, as it occurs in the macromolecular complex, influence its biological properties.

Although polysaccharides and polysaccharide complexes of bacterial origin have been studied extensively (15, 21, 22), none has been shown to produce a toxic effect similar to the one under study. The unique features of this reaction include the multinodular lesion, its rather long duration after a single injection, and the remittent and intermittent character of the process. The ability of certain polysaccharides, such as those of the capsule of the pneumococcus, to remain in the tissues of intact animals for long periods of time is well known (23). The effect of the injection of carrageenin, a polysaccharide of plant origin, on skin of guinea pigs has been described recently by Williams (24). It is of interest to note that the effect was evident for at least 4 weeks. However, the histological reaction was not similar to that produced by the streptococcal extract and the reaction was not intermittent or remittent in character. Meier (25) has studied the ability of a wide variety of polysaccharides to stimulate granulomatous reactions in the subcutaneous tissue of rats and guinea pigs. None was observed to produce multinodular lesions and none of the reactions was described as intermittent or remittent. The total duration of the various reactions was not determined in his study.

The unique features of the reaction produced by the polysaccharide-protein complex isolated from the streptococcal cell warrant further study of the role that this material might play in the non-suppurative sequelae of Group A streptococcal infections.

SUMMARY

The toxic cellular component of Group A streptococci, which produces a chronic multinodular lesion of the dermal connective tissue of rabbits, has been isolated by means of differential centrifugation and electrophoresis. The substance appeared to be a macromolecular complex of polysaccharide and protein, the polysaccharide having the serological and chemical characteristics of the group-specific C substance.

Evidence is presented indicating that the polysaccharide moiety is an essential part of the toxic complex. It is suggested that further studies of this material may help elucidate the mechanism of tissue damage in the non-suppurative sequelae of Group A streptococcal infections.

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