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Biological Properties of Streptococcal Cell-Wall Particles

I. Determinants of the Chronic Nodular Lesion of Connective Tissue

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ABSTRACT

SCHWAB, JOHN H. (University of North Carolina, Chapel Hill). Biological properties of streptococcal cell-wall particles. I. Determinants of the chronic nodular lesion of connective tissue. J. Bacteriol. **90**:1405–1411. 1965.—The capacity of cell-wall fragments to induce a chronic remittent nodular lesion after a single injection into rabbit skin varies qualitatively as well as quantitatively among the streptococci. This variation among strains is the result of a summation of several properties of the bacterial cell, some intrinsic and others extrinsic to the cell-wall structure. With some species, the inability to produce this lesion may be related to the susceptibility of cell walls to lysozyme. Other factors defined in this paper include production of hyaluronidase, and association of the cell walls with a component which can affect the interval between injection and appearance of the nodules, called the latent time. Separation of cell-wall fragments from more soluble cell material by centrifugation results in a shorter latent time. Addition of the soluble supernatant fraction back to the cell walls prolongs the latent time and increases the area of lesion involvement. This latter effect is due to a spreading factor present in most cell extracts. Addition of hyaluronidase to isolated cell-wall fragments duplicates the increased lesion area but tends to shorten further the latent time. Thus, the soluble cell extract contains both a spreading factor and a component which prolongs the latent time, and the final influence on the lesion is in part a product of these two activities. The ease and extent of mechanical disintegration of the cell wall can also vary widely among strains and yield cell extracts differing in their content of cell-wall fragments of optimal size.

The mucopeptide-C polysaccharide complex from the cell walls of β-hemolytic streptococci produces a chronic, remittent, multinodular lesion of connective tissue in the skin of rabbits (Schwab, Cromartie, and Roberson, 1959; Cromartie, Schwab, and Craddock, 1960). Recent studies have been concerned with host factors, such as antibodies to certain cell-wall antigens (Schwab and Cromartie, 1960), age and hypersensitivity (Schwab, 1964), and related factors affected by anti-inflammatory agents (Schwab, Am. J. Pathol., *in press*), which influence this tissue response.

As another approach to the understanding of this reaction, streptococcal strains were selected representing high, moderate, or negligible capacity to produce the nodular dermal lesion. These were compared for susceptibility of cell walls to lysozyme, presence of components other than cell-wall fragments which influence the course of the reaction, and structure and toxicity of cell-wall moieties. The structure of cell-wall mucopeptide is being pursued by immunochemical techniques (Abdulla and Schwab, 1965*a*, *b*), and a description of the dermo-necrotoxic properties of mucopeptide was given in a preliminary report (Schwab and Abdulla, Bacteriol. Proc., p. 46, 1965).

MATERIALS AND METHODS

Sonic extracts. Streptococci were grown in Oxoid Todd-Hewitt broth for 18 hr. The cells were washed three times with cold saline, resuspended in 0.067 M phosphate buffer (*p*H 7.0), and disrupted in a Raytheon 9-kc sonic oscillator for 1 hr. The extent of disruption was followed by measuring change in turbidity in a Klett colorimeter with a no. 54 filter. The extract was centrifuged at 27,000 × *g* for 30 min, and the supernatant fluid was filtered through a Millipore HA filter, dialyzed against water, and lyophilized. This crude

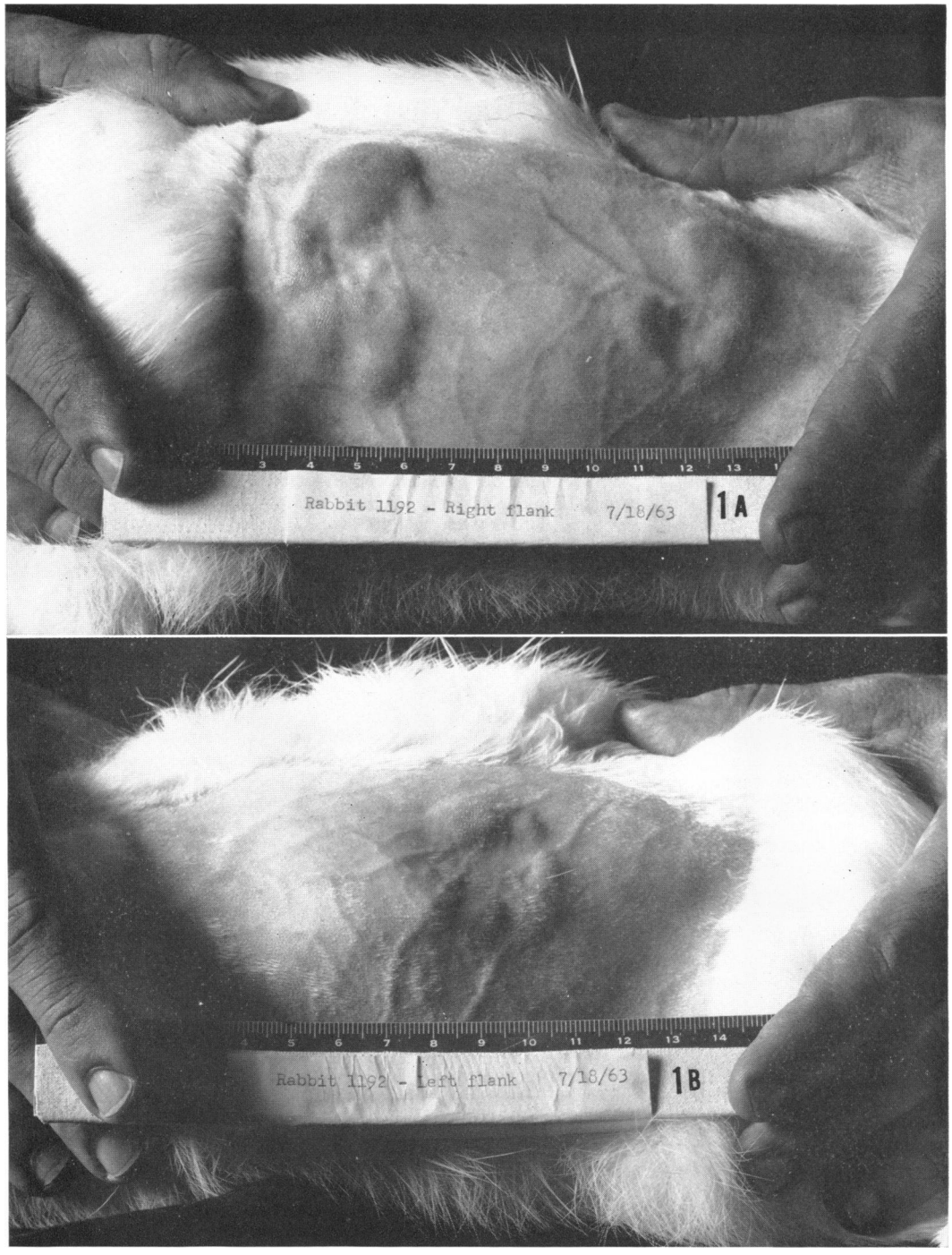

FIG. 1. (A) *Primary nodular lesions produced with group K, strain Turner, streptococcal cell-wall fragments. The rabbit was injected intradermally in the upper flank at two sites. The anterior site (right) received a 90p45-6 cell-wall preparation with no enzyme treatment; 22 μg of nitrogen were injected. The posterior site received a 90p45-6 preparation treated with hyaluronidase and ribonuclease; 15 μg of nitrogen were injected. (B) Opposite flank of same rabbit injected with the same cell-wall preparation treated with hyaluronidase, ribonuclease, and papain; 10 μg of nitrogen were injected. Photographs were taken at 3 days after injection.*

extract contains the soluble components of the cell as well as small fragments of cell wall. Each extract was injected into one of four sites on six rabbits in a dose of 2.0 mg.

Cell-wall fragments (90p45). The small cell-wall fragments were separated from the crude sonic extract by centrifugation in a Spinco no. 40 rotor at 31,500 rev/min (90,000 × *g*) for 45 min. This precipitate is referred to as 90p45, and the supernatant, largely free from cell-wall material, is referred to as 90s45. The precipitate was washed with *p*H 7.0 phosphate buffer three times and labeled 90p45-3. As indicated in Results, some of these fractions were further treated with hyaluronidase, ribonuclease, and trypsin or papain in a concentration of 200 μg/ml at 37 C for 4 hr with a drop of chloroform added. The first two enzymes were added together, followed, after one washing, by the protease. After enzyme treatment, the precipitates were washed an additional three times with buffer, and were labeled 90p45-6.

Cell walls. Washed bacterial suspensions were disrupted in a Mickle shaker with no. 12 Ballantoni glass beads (Salton, 1964). The time required for cell disruption was highly variable for the different streptococcal strains, and was determined by phase microscopy at 5-min intervals. Centrifugation at 500 × *g* for 20 min removed most of the unbroken cells. The large cell walls were collected at 10,000 × *g* in a Servall SS-4 rotor and washed three times with buffer. They were then treated with ribonuclease and trypsin, 200 μg/ml, for 4 hr at 37 C, and were washed an additional three times with water and lyophilized.

Lysozyme treatment of cell walls. Cell walls in a final concentration of 1 mg/ml were incubated with egg white lysozyme (Calbiochem) in a final concentration of 500 μg/ml of *p*H 7.0 tris(hydroxymethyl)aminomethane (Tris) - buffered saline (0.067 M phosphate, 0.04 M NaCl) at 37 C with shaking for 18 hr. After centrifugation at 12,000 × *g* for 30 min, the supernatant fluid was decanted, and the tubes were allowed to drain. The precipitates were suspended with buffer, and rhamnose determinations were made on the precipitate and supernatant fluid. Controls consisted of cell walls in buffer.

Chronic remittent nodular skin lesions. A single intradermal injection of rabbits with sterile cell-wall fragments from certain β-hemolytic streptococci produces a multinodular lesion which appears within 2 to 52 days after injection (Fig. 1). This latent time depends upon several factors, including concentration of cell-wall material, size of cell-wall fragments [large cell walls are not as effective on a weight basis as intermediate cell-wall fragments (Roberson, Schwab, and Cromartie, 1960)], degree of purification of the cell-wall material, and individual animal variation. Another parameter of this lesion is the total area of involvement, which may cover a skin area as large as 70 by 120 mm. With an adequate number of animals in a group, the maximal area of involvement divided by the latent time in days gives a lesion index which is proportional to the dose of cell-wall material (Schwab et al., 1959). This has been a helpful measurement in purification studies, but its quantitative value is limited because of the many factors involved in the evolution of the lesion, as described in this paper.

The primary nodules disappear about 1 to 2 weeks after initial appearance, usually leaving a grossly negative skin area. About 1 to 3 weeks later, a relapse occurs with new nodules appearing within the old area of involvement. The incidence of relapses can be up to 100%, depending on dose and other factors described here. Since there can be considerable individual animal variation, especially with more crude preparations of cell walls, comparative studies are made within one group of animals, insofar as possible.

Chemical analyses. Methods used for determination of nitrogen, rhamnose, and reducing sugar have been described (Schwab et al., 1959).

Results

Comparison of nodular lesion production by various streptococcal strains. Crude sonic extracts were prepared from 10 streptococcal strains representing five serological groups. Intradermal injection of 2.0 mg revealed a spectrum of nodular lesion production between groups and within group C (Table 1).

It is apparent from Table 1 that toxicity of extracts prepared by mechanical disruption may reflect extent of cell breakdown, as measured by turbidity change, and the concentration of cell-wall fragments of optimal size (Roberson et al., 1960), as measured by percentage of cell-wall

Table 1. *Comparison of nodular lesion production with crude extracts of sonic-disrupted streptococci**

Serological group	Strain	Index	Decrease in turbidity†	Percentage of total soluble rhamnose precipitated at 90,000 × *g*
			%	
A	D-58	238	42	84
C	D-10	208	58	43
C	2626	186	—	—
C	H 46-A	112	63	65
C	28 RP 95	46	—	—
C	*Streptococcus equi*	8	73	17
D	*S. durans*	0	—	—
D	F-24	0	—	—
F	Rogers	119	—	—
K	Turner	3	45	—

* Intradermal injection of 2.0 mg of sonic extract.

† Measured as 100 − (optical density after 60 min of sonic vibration/optical density after 0 min of sonic vibration).

TABLE 2. *Comparison of nodular lesion production with cell walls from selected streptococcal strains**

Serological group	Strain	Index/μg of nitrogen	Index/μg of rhamnose
A	D-58	17	3.4
A	C203/29/4	17	3.8
C	H-46 A	15	4.2
C	*Streptococcus equi*	3	1.0
D	F-24	0	0
K	Turner	15	—†

* Intradermal injection of 280 μg.
† This strain contains negligible rhamnose.

rhamnose sedimentable at 90,000 × *g*. A better comparison of the capacity to produce the nodular lesion was obtained with the isolated cell walls from six selected strains (Table 2). Although these preparations are of similar concentration and size, there is still a considerable range of toxic reaction. Therefore, the wide variation between strains is not accounted for as a quantitative difference in cell-wall fragments.

Comparative susceptibility of cell walls to lysozyme. Bacterial suspensions of eight group D strains were surveyed for resistance to lysozyme lysis. Cell walls were prepared from three of these, representing different degrees of susceptibility. A comparison of these with cell walls from four other streptococcal strains shows some inverse relationship between lysozyme effect and lesion production (Table 3). The group A and group C strains which show high resistance to lysozyme are also good lesion producers, whereas the relatively susceptible group D strains produce moderate nodular lesions in an occasional rabbit, only with 1.0 mg, or four times the dose of the other cell-wall preparations. There is no correlation among the group D strains.

It is interesting that the isolated group D cell walls show a relatively high degree of apparent autolysis, in direct proportion to their susceptibility to lysozyme.

Influence of the soluble fraction of sonic extracts on the nodular lesion produced by cell-wall fragments. Separation of cell-wall fragments from the crude sonic extract by differential centrifugation and washing of the sediment yields a cell-wall preparation which consistently shows a shorter latent time than the crude extract. An extreme example of this is shown in Fig. 2 with a group K strain. Nearly all of the nodular lesion-producing activity was associated with the sedimentable cell-wall fraction 90p45-6, and the supernatant 90s45 was relatively inactive. Six rabbits were injected at one of four sites with one of the following preparations: 100 μg of 90p45-6 in buffer, 100 μg of 90p45-6 plus a volume of 90s45 approximately equivalent to the ratio of the two fractions in the crude extract, the same dose of 90s45 plus buffer, and 2.0 mg of crude sonic extract. As seen in Fig. 2, the separated cell-wall fraction 90p45-6 was considerably more active than 20 times the weight of crude extract. Adding the supernatant back to the cell-wall fraction significantly reduced activity. This is further demonstrated by observations collected in Table 4, in which cell-wall fractions of various degrees of purification from several strains were mixed with the relatively nontoxic supernatant fluid

TABLE 3. *Correlation of lysis of isolated cell walls by egg white lysozyme with capacity to produce nodular lesions in rabbit skin*

Streptococcal group	Strain	Primary nodular lesion		Lysozyme digestion	
		Cell wall injected	Index	Per cent rhamnose solubilized*	
				Control	Plus lysozyme
		μg			
A	D-58	250	260	0	0
A	C203/20/4	250	423	0.3	3.5
C	H46A	250	379	0.3	1.6
C	*Streptococcus equi*	250	67	0.6	4.5
D	E-65	1,000	21	5.0	55.0
D	E-1	1,000	9	8.0	69.0
D	F-24	1,000	34	11.0	95.0

* Per cent rhamnose in supernatant fluid after centrifugation at 12,000 × *g* for 30 min.

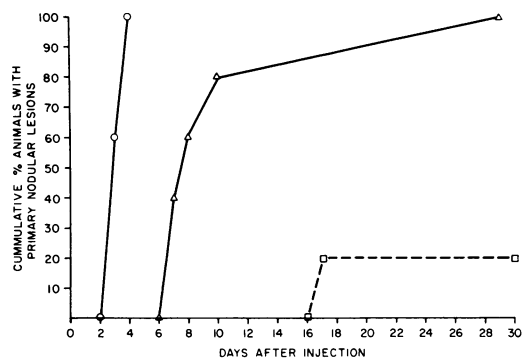

FIG. 2. *Effect of soluble supernatant fraction of sonic extract on latent time between injection of cell-wall fragments and appearance of primary nodular lesions. Cell-wall fragments, 90p45-6, plus buffer, ○; cell-wall fragments plus supernatant, 90s45, △; crude cell extract before separation of cell wall fragments, □. Group K, strain Turner, streptococcus.*

from centrifugation of disrupted cells (90s45). A prolongation of the period between injection and appearance of primary nodules is consistently observed. The effect is more pronounced as the degree of purification of the cell-wall fragments increases. Thus, the electrophoretically purified fraction 1 (Schwab, 1965), from the cell-wall fragments of the D-10, group C strain had a 50% latent time of 2.2 days. This was prolonged to 14 days when mixed with the supernatant fluid. It is quite remarkable that after injection of a few micrograms of this purified cell-wall preparation the skin can remain grossly negative for as long as 22 days and then develop a 3+ lesion 60 by 70 mm in the area around the injection site. It is noteworthy that addition of the group C, *S. equi* supernatant fluid to the group A cell walls modified the latent period of the nodular lesion with an effect comparable to that obtained with the supernatant from the group A extract. The only influence of the supernatant on the incidence of relapses was seen in experiments with the electrophoretically purified cell-wall fragments, which will be treated more fully in the accompanying paper (Schwab, 1965).

Effect of a spreading factor on the nodular lesion. Although mixing the cell-wall fragments with the soluble cell fraction prolongs the latent time, it also increases the area of lesion involvement (Table 4). This reflects the presence of a spreading factor, which is also indicated by the rapidity with which the initial injection volume disappears. The possible influence of spreading factor on the course of the nodular lesion was investigated by treatment of cell-wall fragments with hyaluronidase. Table 5 compares the effect of hyaluronidase and the supernatant, 90s45, on the lesion produced with cell-wall fragments, 90p45, from group C, strain H46-A streptococci. A control of hyaluronic acid plus cell-wall fragments was also included. The shortened latent times obtained in this experiment with hyaluronidase and hyaluronic acid are not significantly different from the control. However, the increased latent period upon addition of supernatant fluid is significant at the 5% level of

TABLE 5. *Effect of hyaluronidase and supernatant fluid (90s45) from sonic extract on primary nodular lesions produced with cell-wall fragments**

Sample†	Mean latent period	*P* vs. buffer control	50% latent period	Mean area	*P* vs. buffer control
	days		*days*	*mm²*	
Cell walls plus buffer	3.0	—	2.5	838	—
Cell walls plus hyaluronidase	2.8	>0.2	2.2	2,100	0.06
Cell walls plus hyaluronic acid	2.6	>0.1	2.1	1,189	>0.1
Cell walls plus supernatant, 90s45	4.4	<0.05	3.7	2,700	0.05

* Cell-wall fragments, 90p45-3 fraction, from group C, strain H-46A streptococci.

† Hyaluronidase and hyaluronic acid in a final concentration of 100 μg/ml, 90s45 added to approximate ratio to cell walls in crude extract. All mixtures held at 37 C for 3 hr.

TABLE 4. *Effect on latent period and area of lesion produced by the cell-wall fragments of addition of supernatant fluids (90s45) from sonic extracts to cell-wall preparations of differing purification*

Cell-wall prepn	Added before injection	50% latent time	Mean latent time*	Mean lesion area
		days	*days*	*mm²*
Group A, D-58, 90p45-1	Buffer	3.6	4.6	912
Group A, D-58, 90p45-1	Group C, *Streptococcus equi* supernatant fluid	5.8	9.0	1,020
Group A, D-58, 90p45-2	Buffer	3.0	3.0	1,162
Group A, D-58, 90p45-2	Group A, D-58 supernatant fluid	5.0	5.6	3,399
Group K, 90p45-6†	Buffer	2.8	3.4	716
Group K, 90p45-6†	Group K supernatant fluid	7.6	12.0	1,050
Group C, D-10, 90p45-6, F-1‡	Buffer	2.2	2.6	824
Group C, D-10, 90p45-6, F-1‡	Group C, D-10 supernatant fluid	14.0	13.8	2,517

* By Student's *t* test, all of the differences in latent time between cell-wall fragments plus supernatant fluids or plus buffer have a *P* value of 0.05 or less.

† Treated with hyaluronidase and ribonuclease.

‡ Purified by sucrose gradient column electrophoresis.

TABLE 6. *Influence of hyaluronidase on nodular lesions produced with cell-wall fragments, showing differentiation between effect on bacterial or tissue substrate*

Group	Procedure*	Mean latent time	*P* vs. contfol	Mean area	*P* vs. control	Relapse, positive/total
		days		*mm²*		
I	Cell walls + buffer, 37 C, 3 hr, wash twice	4.7	—	396	—	4/7
II	Cell walls + enzyme, 37 C, 3 hr, wash twice	5.1	>0.2	709	0.016	3/7
III	Cell walls + buffer, 4 C, wash twice	5.3	—	351	—	3/7
IV	Cell walls, 4 C, wash twice, plus enzyme	3.1	0.06	1,038	0.016	5/7

* Cell-wall fragments, 90p45-3 fraction, from group C strain D-10 streptococcus, 5 μg of rhamnose injected. Hyaluronidase final concentration, 100 μg/ml; 20 μg injected in 0.2-ml volume.

TABLE 7. *Effect of enzyme treatment of cell-wall fragments on the production of remittent nodular lesions*

Sample*	Nitrogen injected	Primary nodular lesion				Relapse, no. positive/total
		Mean latent time	Mean area	Mean index	Mean index/μg of nitrogen	
	μg	*days*	*mm²*			
90p45-6, no enzyme	22	3.8	919	327	15	5/6
90p45-6 + hyalurinidase, ribonuclease	15	3.5	1,560	571	38	5/6
90p45-10 + hyaluronidase, ribonuclease, papain	10	4.2	1,104	370	37	4/6

* Cell-wall fragments, 90p45 fraction, from group K, strain Turner streptococci.

confidence, and the increased area of lesion involvement with both hyaluronidase and supernatant fluid is also significant.

The experiment was repeated to determine whether the spreading factor was acting on bacterial components, or on a tissue substrate after injection. Treatment of cell-wall fragments from group C, strain D-10 streptococci with hyaluronidase, followed by washing to remove most of the enzyme, had no effect on the incidence of relapses and did not shorten the latent period (groups I and II, Table 6). However, injection of cell-wall fragments immediately after mixing with hyaluronidase without incubation or washing, did increase the latent period as well as decrease the total area of involvement (groups III and IV, Table 6).

Analysis of wash supernatant fluids and sediments showed 33% of total reducing sugar solubilized by hyaluronidase, compared with 5.7% in the control.

In addition to the studies with hyaluronidase, several experiments with different streptococcal strains have included treatment of cell-wall fragments with papain, trypsin, or ribonuclease (Table 7, *also* Schwab, 1965).

DISCUSSION

A single intradermal injection of rabbits with cell-wall fragments from many, but not all, streptococcal strains produces a chronic remittent nodular lesion of connective tissue. These studies provide further understanding of the mechanism of this experimental lesion.

Many factors influence the tissue response to cell walls, and thus the nature of the reaction to extracts of cells (and presumably to cells being disposed of in vivo after infection) reflects a summation of properties. Some of these factors are properties of the host, such as neutralizing antibody (Schwab and Cromartie, 1960), and age and hypersensitivity (Schwab, 1964). Cogent properties of the bacterial cell which have been partially defined include: susceptibility of cell walls to lysozyme or other unknown tissue muramidases, quantitative or structural association of cell walls with certain soluble cell components, and production of hyaluronidase. In addition, there are properties related to the size of cell-wall particles (Roberson, Schwab, and Cromartie, 1960).

The capacity to induce the nodular lesion

seems related to the extremes of susceptibility to egg white lysozyme. That is, those cell walls which are very resistant to lysozyme are the most effective lesion producers, whereas the isolated mucopeptide and those cell walls which are very susceptible to lysozyme do not readily evoke this lesion. However, one would expect that partially susceptible cell walls would be intermediate in toxicity, which does not seem to be the case. Possibly, even minimal lysis is sufficient to hasten elimination of cell-wall material from tissue, or otherwise "detoxify" the active complex of the cell wall. Another unknown factor in interpretation is the comparative action of egg white lysozyme and mammalian tissue lysozyme.

Although all of the nodular lesion-producing activity is associated with isolated cell-wall fragments, some more soluble component in cell extracts has a significent influence on the course of the reaction. Separation of cell-wall fragments from the crude cell extract before injection consistently results in a shortened latent time. Addition of the supernatant fluid back to the cell-wall sediment again prolongs the latent period between injection and appearance of nodules.

Some of this activity remains associated with the cell-wall fragments, and, although repeated washing reduces the amount present, it cannot be eliminated by differential centrifugation alone.

Results of enzyme studies show that this activity is not a substrate of papain, trypsin, ribonuclease, or hyaluronidase, since incubation of the cell-wall fragments with these enzymes followed by washing to remove enzyme does not significantly alter the latent time or incidence of relapses.

Mixing of the soluble cell fraction back with the cell-wall fragments has another effect in addition to prolonging the latent time, namely, the area of lesion involvement is much larger compared with that obtained with isolated cell walls. This is due to a spreading factor, probably hyaluronidase, present in most cell extracts. However, the spreading factor is not responsible for the prolonged latent time, since injection of purified hyaluronidase with the cell-wall fragments not only increases the area of involvement, but tends to shorten the latent time. Thus, the production of hyaluronidase by a bacterial strain can be an important factor in the course of this experimental lesion of connective tissue, and conceivably could be of significance in tissue alterations associated with cell disposal after infection.

It is evident that the influence of the soluble cell fraction on the tissue reaction to cell walls is a balance of two effects. The hyaluronidase tends to extend the lesion and shorten the latent time, perhaps by facilitating contact of cell walls with tissue sites. On the other hand, another factor tends to lengthen the latent time, perhaps by preventing the contact of cell walls with tissues.

ACKNOWLEDGMENT

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LITERATURE CITED

ABDULLA, E. M., AND J. H. SCHWAB. 1965*a*. Immunological properties of bacterial cell wall mucopeptides. Proc. Soc. Exptl. Biol. Med. **118**:359–362.

ABDULLA, E. M., AND J. H. SCHWAB. 1965*b*. Antigenic specificity of bacterial cell wall mucopeptides. Federation Proc. **24**:186.

CROMARTIE, W. J., J. H. SCHWAB, AND J. G. CRADDOCK. 1960. The effect of a toxic cellular component of group A streptococci on connective tissue. Am. J. Pathol. **37**:79–99.

ROBERSON, B., J. H. SCHWAB, AND W. J. CROMARTIE. 1960. Relation of particle size of C polysaccharide complexes of group A streptococci to toxic effects on connective tissue. J. Exptl. Med. **112**:751–764.

SALTON, M. R. J. 1964. The bacterial cell wall. Elesevier Publishing Co., New York.

SCHWAB, J. H. 1964. Analysis of the experimental lesion of connective tissue produced by a complex of C polysaccharide from group A streptococci. II. Influence of age and hypersensitivity. J. Exptl. Med. **119**:401–408.

SCHWAB, J. H. 1965. Biological properties of streptococcal cell-wall particles. II. Purifications by density gradient column electrophoresis. J. Bacteriol. **90**:1412–1419.

SCHWAB, J. H., AND W. J. CROMARTIE. 1960. Immunological studies on a C polysaccharide complex of group A streptococci having a direct toxic effect on connective tissue. J. Exptl. Med. **111**:295–307.

SCHWAB, J. H., W. J. CROMARTIE, AND B. ROBERSON. 1959. Identification of a toxic cellular component of group A streptococci as a complex of group-specific C polysaccharide and a protein. J. Exptl. Med. **109**:43–54.