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Biological Properties of Streptococcal Cell-Wall Particles

II. Purification by Density Gradient Column Electrophoresis

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ABSTRACT

SCHWAB, JOHN H. (University of North Carolina, Chapel Hill). Biological properties of streptococcal cell-wall particles. II. Purification by density gradient column electrophoresis. J. Bacteriol. **90**:1412–1419. 1965.—Sucrose gradient column electrophoresis was utilized as a preparative method to purify streptococcal cell-wall fragments. Enzyme-treated cell-wall preparations could be resolved into two major fractions. All of the cell-wall material was in the fraction of slower electrophoretic mobility. The latent time between injection and appearance of nodular lesions in rabbit skin was significantly shorter with this fraction, compared with crude material. In addition, the incidence of relapses of the nodular lesion was reduced with this purified preparation. Addition of the faster electrophoretic fraction back to the slower cell wall-containing fraction prolonged the latent time between injection and appearance of nodules, and increased the incidence of relapses. The latent time was directly proportional to the ratio of the two fractions. Antigens of the electrophoretic fast fraction were distinct from the C polysaccharide and mucopeptide antigens of the cell wall.

The procedure of Salton and Horne (1951) for separation of bacterial cell walls has resulted in identification of components peculiar to this structure, and comparison of the chemical composition of cell walls from a wide variety of bacteria (Salton, 1964). Electron microscopy is usually the criterion of purity of these preparations. While this is satisfactory for purposes of chemical analysis, it is not adequate when attempting to associate biological properties with the cell wall (Roberson and Schwab, 1960, 1961).

We have previously demonstrated that cell-wall preparations purified by differential centrifugation and sucrose gradient centrifugation were heterogeneous electrophoretically as well as antigenically (Roberson and Schwab, 1960), but it could not be determined whether this reflected contamination with noncell-wall material. In attempting to elucidate the mechanisms of the inflammatory reactions to streptococcal cell walls, it was apparent that several factors, some intrinsic and others extrinsic to the cell-wall structure, determine the course of the tissue response (Schwab, 1965). Therefore, it was of importance to develop preparative methods for obtaining more homogeneous material.

Because of the particulate nature of cell walls, methods of purification have previously been limited to centrifugation techniques. This paper reports the application of sucrose gradient column electrophoresis to the purification of cell-wall preparations. The method allows separation of the resolved components on a preparative scale, and demonstration of their significance in tissue reactions.

MATERIALS AND METHODS

Cell-wall fragments. The collection of bacterial cells and preparation of cell walls and cell-wall fragments (90p45) were described in the previous paper (Schwab, 1965). The label 90p45-3 refers to cell-wall fragments in the precipitate after centrifugation of sonically disrupted cells at 90,000 × *g* for 45 min, followed by three washings with 0.067 M phosphate buffer (*p*H 7.0). Some preparations were further treated with ribonuclease and hyaluronidase, 200 μg/ml, for 3 hr at 37 C, followed by one washing and then treatment with trypsin, 200 μg/ml, and finally were washed three times with buffer and labeled 90p45-6.

Moving-boundary electrophoresis of cell-wall fragments. Cell-wall fragments (90p45-3) in a concentration of approximately 0.4% were dialyzed against Γ/2 0.1 phosphate buffer (*p*H 7.7) and electrophoresed in an 11-ml Tiselius cell in a Spinco model H apparatus as previously described (Schwab, Cromartie, and Roberson, 1959). Figure

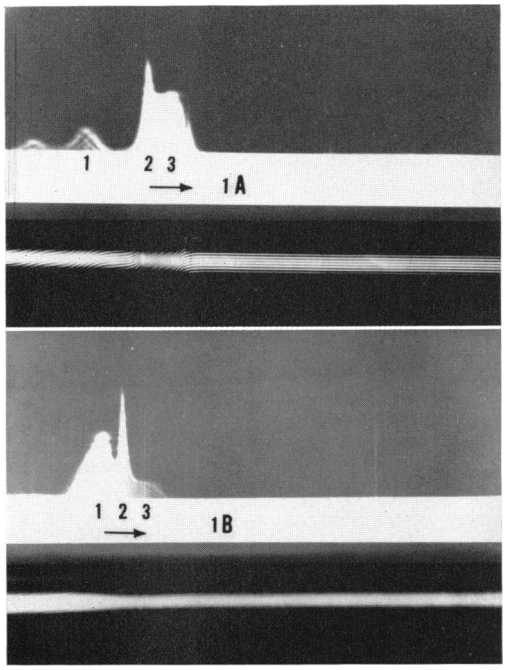

FIG. 1. *Moving-boundary electrophoresis of cell-wall fragments 90p45-3. (A) Group C strain H 46-A streptococcus after 61.4 min, 6.0 v/cm. (B) Group A strain D-58, 45 min.*

1 shows the resolution of three components after 1 hr at 6.0 v/cm and 13 ma. The slow fraction 1 was removed by needle after 3 hr from the descending limb and subsequently a middle fraction, 1 plus 2, was removed. The fast fraction, 2 plus 3, was removed from the ascending limb of the Tiselius cell.

Purification of cell walls and cell-wall fragments by density gradient column electrophoresis. An LKB electrophoresis column was used with a sucrose gradient formed with reagent-grade sucrose in Γ/2 0.05 phosphate buffer (pH 7.7). After filling the right electrode vessel (anode) with 50% sucrose in buffer up to 2 cm into the center column, a gradient was formed in the center column from the mixing vessels with 50% sucrose in the bottom and buffer in the upper reservoir. The method of filling was according to Svensson, as described by Bloemendal (1963), for ascending electrophoresis. Approximately 50 mg of cell-wall fragments 90p45-6, which had been treated with ribonuclease, hyaluronidase, and trypsin, were dialyzed against 40% sucrose, and the final volume was adjusted to 5 to 7 ml. After equilibrating the density to that at the capillary tip, the sample was introduced by gravity flow, rinsing the tubing with sucrose of the same density. A potential at the electrodes of 280 v and a current of 12 ma was applied for 11 to 20 hr. All separations were done at 20 C.

At the conclusion of the run, two zones of turbidity could be seen with oblique lighting. The leading edge of the fastest material moved 15 cm in 11 hr. Below this was a clear area of 5 cm, separating the upper band from the lower turbid area, which had moved 7 cm above the capillary tip. The column contents were collected by gravity flow in 5-ml fractions at 1.5 ml/min with a fraction collector. The tube contents were measured by absorption at 320, 280, and 260 mμ with the resolution shown in Fig. 2 and 3, after correction for sucrose. The tubes were pooled into fractions 1, 2, and 3 as indicated in Fig. 2 and 3, dialyzed against distilled water for 5 days with toluene added to the water, and lyophilized. The distribution of recovered rhamnose, nitrogen, and reducing sugar among the pooled fractions is shown in Table 1. Nearly all of the rhamnose, the sugar characteristic of streptococcal cell walls, is in fraction 1.

Large cell-wall preparations were also studied by column electrophoresis. These were obtained by disrupting cells with glass beads in a Mickle apparatus (Salton, 1964), washing by differential centrifugation, and further purifying by sucrose gradient centrifugation (Roberson and Schwab, 1960). These suspensions always clumped in the electrophoresis column, but since they did not settle too far down in the column they could be recovered. Two fractions of faster electrophoretic mobility separated from the cell walls in low concentration. One of these fractions was similar antigenically to the fast fraction 3 resolved from the cell-wall fragments, 90p45.

Antigenic studies. Immunodiffusion was done in

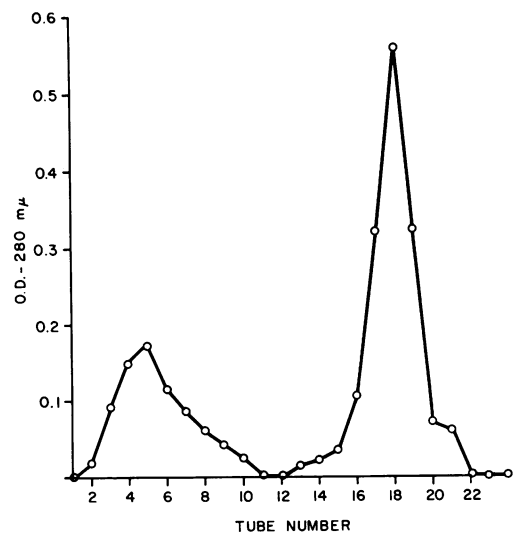

FIG. 2. *Effluent from sucrose gradient column electrophoresis of group C strain D-10 cell-wall fragments 90p45-6 treated with ribonuclease, hyaluronidase, and trypsin. Ascending electrophoresis 11 hr, movement to the right. Fraction 1 (slow) pool, tubes 2 to 10; fraction 2 (middle) pool, tubes 11 to 14; fraction 3 (fast) pool, tubes 15 to 21.*

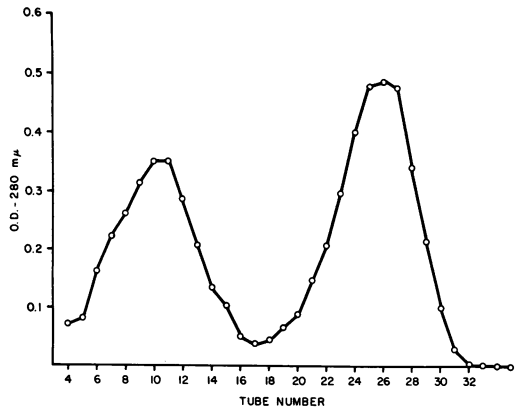

FIG. 3. *Effluent from sucrose gradient column electrophoresis of group A strain D-58 cell-wall fragments 90p45-6 treated with ribonuclease, hyaluronidase, and trypsin. Ascending electrophoresis 19 hr movement to the right. Fraction 1 (slow) pool, tubes 5 to 15; fraction 2 (middle) pool, tubes 16 to 19; fraction 3 (fast) pool, tubes 20 to 31.*

TABLE 1. *Distribution of rhamnose, nitrogen, and reducing sugar in fractions recovered from sucrose gradient column electrophoresis of cell-wall fragments from a strain D-10 group C streptococcus*

Fraction	Percentage of the total recovered		
	Rhamnose	Nitrogen	Reducing sugar*
1 (slow)	93	69	33
2 (middle)	2	6	30
3 (fast)	5	25	37

* Less contribution of rhamnose.

0.5% Ionagar in borate buffer (*p*H 8.2) by the Ouchterlony (1949) method. Rabbit antisera to cell-wall components were obtained by intravenous injection of heat-killed, trypsin-treated vaccines according to the method of McCarty and Lancefield (1955). Antibody to the fast electrophoretic fraction 3 was obtained from rabbits immunized in the foot pad with the antigen in complete Freund's adjuvant (Difco). Antiserum was also obtained from rabbits immunized intradermally at weekly intervals with the heat-killed streptococcal vaccine. This contained antibodies to certain cell components, including mucopeptide, but lacked antibodies to group-specific C polysaccharide.

Tissue reactions. Chronic, multinodular, relapsing lesions are produced after a single injection of cell-wall fragments into rabbit skin. This response is described in detail elsewhere (Schwab, 1965; Cromartie, Schwab, and Craddock, 1960). The pertinent point for this paper is that the latent time between injection of cell walls and appearance of nodules, as well as the incidence of relapsing lesions, is determined by concentration and size distribution of cell-wall fragments, and also another cell component associated with the soluble fraction of cell extracts. The lesion index is the maximal lesion area divided by time (in days) required for appearance of nodules.

RESULTS

Moving-boundary electrophoresis of cell wall fragments. The electrophoretic pattern of cell-wall fragments (90p45-3) from a group C strain H46-A streptococcus is shown in Fig. 1A. A pattern from a group A, strain D-58, is shown also. The difference in mobility between fractions 1 and 2 is greater in the group C than in the group A strain, facilitating separation of the slowest component. As previously reported for the group A strains (Schwab et al., 1959), nearly all of the cell-wall material and toxic activity was in the slow component, labeled fraction 1 (Table 2). Upon intradermal injection, this fraction was four to five times as toxic as the starting material on a weight basis. This was an absolute increase in activity per microgram of rhamnose, which was not relatable to the mere removal of inert material. Fraction 1 plus 2 was less active, and the fast fraction, 2 plus 3, was almost devoid of capacity to produce the nodular lesion (Table 2).

In spite of the fact that isolated fraction 1 was more active in producing the primary nodular lesion, the incidence and size of the relapsing lesion was much reduced. This was investigated further in a second group of rabbits injected on the same flank at two sites with either fraction 1 plus buffer or fraction 1 mixed with an equal volume of the fast fraction just prior to injection (Table 2). With this smaller dose, no relapses were seen over a period of 35 days at sites receiving the fraction 1 alone, whereas at an adjacent site receiving the mixture of fraction 1 plus fast fraction, three of five rabbits developed relapses.

That the fastest electrophoretic component interferes with the initial nodular lesion development is shown by comparing the latent time of fraction 1 plus buffer (1.6 days) with that of fraction 1 plus fast fraction (5.0 days). The reconstituted mixture of fast plus slow components has a lesion-producing capacity of the same order as the 90p45-3 starting material.

The same moving-boundary electrophoretic separation of a 90p45 cell-wall fraction was repeated with another group C strain, D-10 (Table 3). In this experiment, the isolated frac-

TABLE 2. *Comparison of nodular lesion production by moving-boundary electrophoretic fractions of cell-wall fragments from a group C strain H46-A streptococcus*

Sample	Primary nodular lesion					Relapse		Amt injected (μg)		
	50% latent time†	Mean latent time	Mean area	Mean index	Index/μg of rhamnose	Positive/total	Mean area	Rhamnose	Nitrogen	Reducing sugar
	days	*days*	*mm²*				*mm²*			
CWF (90p45-3)*	3.8	4.2	1,017	270	7.5	2/5	292(2+)	36	13	12
Slow (1)	1.6	2.2	1,196	554	35	1/5	45(±)	16	4	1.5
Middle (1 + 2)	2.2	2.6	857	340	19	2/5	160(2+)	18	4.4	4.8
Fast (2 + 3)	—	19‡	98	5	1.5	0/5	0	2.8	2.4	6.8
CWF + papain (90p45-6)	3.5	4	348	93	8	3/5	171(1+)	11	2.7	3.4
Slow + buffer	1.6	2.2	957	444	55	0/5	0	6.5		
Slow + fast	5.0	7.2	513	94	12	3/5	197(2+)	7.9		

* Cell-wall fragments, 90p45-3 fraction from sonic extract, was the starting material subjected to electrophoresis.

† Time required for 50% of animals to show nodular lesion. Obtained from plot of percentage of animals positive against time after injection.

‡ Two of five rabbits eventually reacted to this fraction, compared with five of five with other fractions.

TABLE 3. *Comparison of nodular lesion production by moving-boundary electrophoretic fractions of cell-wall fragments from group C strain D-10*

Sample	Rhamnose injected	Primary nodular lesion					Relapse, no. positive/total
		50% latent time	Mean latent time	Mean area	Mean index	Index/μg of rhamnose	
	μg	*days*	*days*	*mm²*			
CWF (90p45-3)* + buffer	11	2.7	3.6	660	256	23	4/5
CWF (90p45-3) + fast (2 + 3)	11	3.2	4.2	736	218	20	4/5
Slow (1) + buffer	4	2.2	2.6	824	339	85	0/5
Slow + fast	4	3.8	6.8	528	99	25	4/5
Fast + buffer	1	17†	14	106	7	7	0/5

* Cell-wall fragments, 90p45-3 fraction from sonic extract, was the starting material subjected to electrophoresis.

† Three of five rabbits eventually reacted to this fraction, compared with five of five with other fractions.

tion 1 was seven times more effective on a weight basis than the starting material, and no relapses occurred during 35 days of observation. Again, adding the fast fraction back with the cell-wall fraction not only prolonged the time required for development of primary nodules, but also restored the incidence of relapses. Addition of the fast fraction to the 90p45 starting material, to give an excess of this component, had a less pronounced effect on the latent time.

Sucrose gradient column electrophoresis of cell-wall fragments. Because the small amount of material isolated from a Tiselius cell limits the studies which can be done, and also because of the technical difficulties in recovering "clean" fractions from this cell, the above results were confirmed and extended by use of electrophoretic separation in a density gradient column. A 90p45 fraction from the D-10, group C strain was prepared as in the previous experiments, except that it was treated with ribonuclease, hyaluronidase, and trypsin, and was washed an additional three times. The distribution of material as shown in Fig. 2 is comparable to the boundary pattern obtained in a Tiselius cell after similar enzyme treatment. In the density gradient column, trypsin treatment of the sample is essential to achieve good resolution of the slow and fast fractions.

Six rabbits were injected intradermally at

one of four sites with the fast fraction 3, middle fraction 2, or the slow fraction 1 in two concentrations, as indicated in Table 4. Fraction 1 produced typical primary nodular lesions in a dose of 0.64 μg of nitrogen. The minimal effective dose was not determined. Fraction 2, although an equivalent amount of rhamnose and three times as much nitrogen was injected, produced only a very moderate reaction, apparently because of contamination with the fraction 3. Fraction 3 produced no nodular reaction. No relapses were seen at any of the sites over a period of 30 days, even with the highest dose of the purified slow fraction.

A second group of nine rabbits was then injected intradermally at four sites with each one of the mixtures of fraction 1 plus fraction 3 indicated in Table 5. The fractions were at room temperature and were injected within 20 min after mixing. The results in Table 5 clearly show that electrophoretic fraction 3, which has no toxicity itself, influences the incidence of relapsing nodules produced with fraction 1. Fraction 3 also determines the latent period between time of injection and gross appearance of nodules (Fig. 4). This suppressive effect is in direct proportion to the ratio of fraction 3 to fraction 1. Since the amount of fraction 1 used in this experiment is five times the smaller dose shown in the previous experiment to be very effective in producing nodular lesions, it is assumed the 33% incidence of relapses with fraction 1 indicates that it is still contaminated with fraction 3. This is confirmed by immunological studies with antibody specific for fraction 3, which is discussed below. The cell-wall preparation introduced into the column had a latent period and incidence of relapses comparable to the mixture of the two major fractions 1 and 3 which approximated their ratio in starting material (Fig. 4).

The results were repeated with a group A streptococcus, strain D-58. The starting material on the sucrose gradient column was a 90p45 fraction treated with hyaluronidase, ribonuclease, and trypsin. The distribution of components (Fig. 3) is similar to that observed with the group C strain. The pooled tubes were dialyzed, lyophilized, and suspended in buffered saline. In this experiment, fraction 3 was kept constant and mixed with two concentrations of fraction 1. Nine rabbits were injected intradermally at one of four sites with the mixtures indicated in Table 6 and Fig. 5.

TABLE 4. *Comparison of nodular lesion production by density gradient electrophoresis fractions of cell-wall fragments from group C strain D-10*

Fraction	Amt injected		Primary Nodular lesion					Relapse, no positive/total
	Rhamnose	Nitrogen	50% latent time	Mean latent time	Mean area	Mean index	Index/μg of rhamnose	
	μg	μg	days	days	mm^2			
Slow (1)	10.7	3.2	2.0	4.2	360	222	21	0/6
Slow (1)	2.1	0.64	2.0	6.0	273	133	63	0/6
Middle (2)	2.0	2.2	5.0	5.0	55	11	6	0/6
Fast (3)*	1.2	2.4	—	—	0	0	0	0/6

* Zero of six animals reacted within 35 days, compared with six of six with other fractions.

TABLE 5. *Effect of electrophoretic fraction 3 on latent time and incidence of relapsing nodular lesions produced by fraction 1**

Sample	Ratio of fraction 3 to fraction 1	Rhamnose injected	Primary nodular lesion		Relapses, per cent animals positive	
			50% latent time	Mean latent time	First	Second
		μg	days	days		
Fraction 1 + buffer	0	10.7	1.8	2.3	33	12
Fraction 1 + fraction 3	1	10.7	2.9	4.1	87	50
Fraction 1 + fraction 3	3	10.7	5.8	8.2	75	25
CWF (90p60-6)*	—	13	2.6	4.4	75	62

* Cell-wall fragments, 90p45-6 fraction, from group C strain H46-A streptococcus, subjected to sucrose gradient column electrophoresis.

The results again show the direct relationship of concentration of fraction 1 to the latent period and to the incidence of relapses, and the obvious capacity of fraction 3 to prolong the latent period required for appearance of primary nodules and at the same time to increase the incidence of relapses. It is also observed that over a fivefold increase in concentration of fraction 1, the addition of fraction 3 still caused an approximately threefold increase in 50% latent time.

Antigenic analysis of electrophoretic fractions. The fast fraction 3 did not react in immunodiffusion plates with rabbit serum containing antibody to streptococcal cell-wall antigens. However, immunization of rabbits with isolated fraction 3 gave an antiserum showing three precipitin lines with the homologous antigen. The most pertinent immunological reactions have been incorporated into one plate (Fig. 6). The slow fraction 1 in the central well shows two precipitin lines with antigroup A cell-wall serum (well 1). Absorption of this antiserum with isolated C polysaccharide removed one line (well 2). Absorption with group A mucopeptide (well 3) eliminated the sharp line close to the central well, which is apparently an integral antigenic moiety of the cell-wall mucopeptide (Abdulla and Schwab, 1965*a*). A line of identity is seen with anti group C serum (well 4) which agrees with other studies on the antigenic relationship of certain bacterial mucopeptides (Abdulla and Schwab, 1965*b*). Antiserum against fraction 3 (well 5) does not show this mucopeptide reaction, but does give three different lines of reaction, confirming that there is still some contamination of fraction 1 with fraction 3. The antiserum to fraction 3 also shows three lines of identity with certain electrophoretic fractions of the supernatant fluid (90s45).

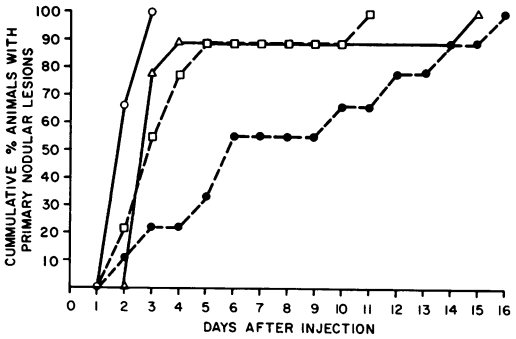

FIG. 4. *Comparison of the latent time required for appearance of nodular lesions after injection of electrophoretic fraction 1 alone, ○; fraction 1 plus 1 volume of fraction 3, □; fraction 1 plus 3 volumes of fraction 3, ●; and intact cell-wall fragments, △. Fractions obtained from sucrose gradient column electrophoresis of group C, strain D-10 cell-wall fragments, 90p45-6.*

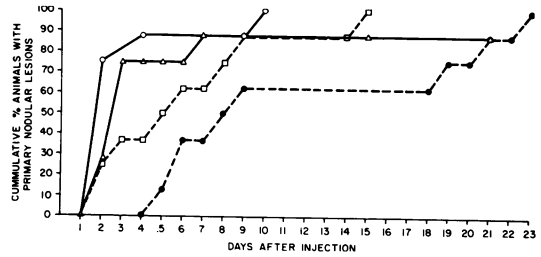

FIG. 5. *Effect of concentration of fraction 1 on the ability of fraction 3 to prolong the latent time required for appearance of nodular lesions. Fraction 1 alone, 30 μg of rhamnose, ○; fraction 1, 30 μg of rhamnose, plus 1 volume fraction 3, □; fraction 1 alone, 6 μg of rhamnose, △; fraction 1, 6 μg of rhamnose plus 1 volume of fraction 3, ●. Fractions obtained from sucrose gradient column electrophoresis of group A, strain D-58 cell-wall fragments, 90p45-6.*

TABLE 6. *Effect of electrophoretic fraction 3 on latent time and incidence of relapses produced by fraction 1**

Sample†	Rhamnose injected	Primary nodular lesion		Relapses, per cent		
		50% latent time	Mean latent time	Animals positive		
				First	Second	Third
	μg	*days*	*days*			
Fraction 1 + buffer	30	1.7	3.2	57	14	0
Fraction 1 + fraction 3	30	5.0	6.2	86	43	14
Fraction 1 + buffer	6	2.5	5.6	28	0	0
Fraction 1 + fraction 3	6	8.0	12.1	57	0	0

* Cell-wall fragments, 90p45-6, from group A strain D-58 streptococcus, subjected to sucrose gradient column electrophoresis.

† Each mixture in a 1:1 ratio.

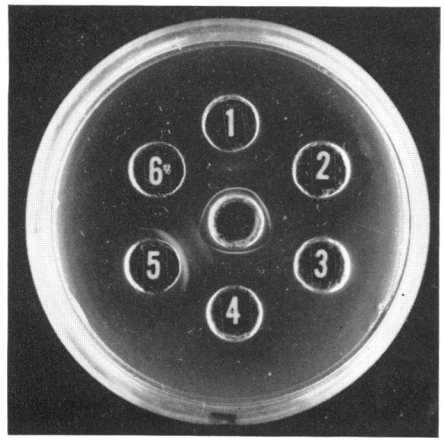

FIG. 6. *Agar diffusion showing distinction of electrophoretic fractions 1 and 3. Central well, fraction 1 derived from cell-wall fragments of group A, strain D-58. Outer wells: 1, antigroup A cell-wall serum; 2, antigroup A cell-wall serum absorbed with group A C polysaccharide; 3, antigroup A cell-wall serum; 5, antifast fraction (3); 6, antiserum from rabbits immunized intradermally with group A streptococcal vaccine.*

DISCUSSION

Previous studies have demonstrated that streptococcal cell-wall fragments can produce a chronic remittent nodular lesion of dermal connective tissue after injection into rabbit skin (Schwab et al., 1959; Cromartie et al., 1960). Although only the cell-wall fraction can produce this lesion, soluble cell components can influence the latent time between injection and appearance of nodules (Schwab, 1965).

The present experiments demonstrate that the cell-wall fragments, even after repeated washing and treatment with enzymes, are still associated with components having this property. Utilizing moving-boundary or density gradient electrophoresis, enzyme-treated cell-wall fragments can be resolved into two major fractions. Nearly all of the cell-wall material and the lesion-producing activity is associated with the fraction of slower electrophoretic mobility. The nodular lesion produced with this purified fraction has a short latent time between injection and appearance of nodules, and a very consistent response is obtained among individual animals. Mixing the two fractions again prolongs the latent time in direct proportion to their ratio. Addition of the soluble supernatant fraction from the sonic extract to the purified cell-wall fraction also greatly prolongs the latent time.

Another interesting feature of these experiments is the observation that lesions produced with electrophoretically purified cell-wall fragments display a reduced incidence of relapses. Some preparations produced no relapses, although the primary nodules appeared with great consistency at 2 to 3 days. Addition of faster electrophoretic fraction back to the slower cell wall-containing fraction, resulted again in a higher incidence of relapses, similar to that obtained with unresolved cell-wall material.

The fraction resolved from cell-wall particles by electrophoresis is antigenically heterogeneous, but is distinguishable from the major cell-wall antigens. Antigenic analysis provides further evidence that this fraction is primarily a soluble component of cells, which is difficult to dissociate from cell-wall preparations. Chemical analysis of the fast electrophoretic material resolved by moving-boundary electrophoresis (Schwab et al., 1959) has revealed only a relatively high concentration of reducing sugars and protein, not characteristic of the structural components of the cell wall.

The manner in which this material influences the reaction to the cell-wall fraction is unknown. It could conceivably function either by steric hindrance of critical sites on the cell wall or by competition for tissue sites. Another possibility being investigated involves an active pharmacological effect on host responses.

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