Biological Properties of Streptococcal Cell-Wall Particles

III. Dermonecrotic Reaction to Cell-Wall Mucoproteins

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ABSTRACT

ABDULLA, ESSA M. (University of North Carolina School of Medicine, Chapel Hill), AND JOHN H. SCHWAB. Biological properties of streptococcal cell-wall particles. III. Dermonecrotic reaction to cell-wall mucoproteins. J. Bacteriol. 91:374–383. 1966.—Intradermal injection of rabbits and guinea pigs with mucoprotein suspensions produced an acute necrotic lesion which reached maximal severity within 24 hr and gradually subsided with scar formation. Necrosis was evident within 4 hr after injection of 100 μg, and an indurated area (10 × 10 mm) was produced with as little as 5.0 μg. Mucoproteins from six bacterial strains were studied. Comparison of cell walls and derived mucoproteins showed that the acute necrotic lesion tended to be more severe as the residual polysaccharide was decreased. Hyperimmunization with mucoprotein reduced the acute reaction, with evidence of immunological specificity. Incubation with lysozyme also modified the reaction in relation to extent of digestion. Toxicity was related to particle size, since extended sonic vibration decreased activity. Histological sections showed intense accumulations of polymorphonuclear leukocytes, along with altered collagen. A chronic nodular lesion appeared about 7 days after injection of the intact cell-wall fragments. In contrast to the acute necrotic reaction, this lesion was rarely produced by the mucoprotein separated from polysaccharide.

Intradermal injection of a sterile suspension of streptococcal cell-wall fragments into normal rabbits produces an erythema and edema within the first few hours, which subsides by 48 to 72 hr. Within 3 to 16 days after this single injection, a multinodular lesion appears. Over the next few days, the nodules tend to coalesce to form a plaque as large as 10 cm in diameter, which gradually subsides. About 3 weeks after the injection, new nodules appear within the old area of involvement in a high percentage of animals. This process of remission and exacerbation can occur as many as three times over a period of 90 days (5, 17, 18, 19, 20). Several factors have been defined which influence the area of involvement, as well as incidence of relapses and latent time between injection and appearance of primary nodules (17). Necrosis and vascular thrombi are not a prominent feature in any phase of this chronic reaction to the intact cell-wall particles.

To help elucidate the mechanism of this remarkable lesion, the cell walls were separated into two major components, mucoprotein and group-specific C polysaccharide, and the toxic properties of each were studied. Neither of these fractions alone could produce the chronic nodular lesion elicited by the mucoprotein-C polysaccharide complex. However, mucoprotein freed from polysaccharide produced a severe acute necrotic lesion, which is the subject of this paper.

MATERIALS AND METHODS

Organisms. Cell walls and mucoprotein were derived from the following bacteria: group A streptococcus, type 3, strains D58 and C203/29/4; group A variant streptococcus, strain B455, derived from C203/29; group C streptococcus, strain H-46A; group D streptococcus (Streptococcus faecalis), strain F-24; Staphylococcus albus; Chromobacterium violaceum; and Bacillus cereus.

Collection of cells. The bacteria were grown as previously described (18). Cells from an 18-hr broth culture were collected by centrifugation, washed two times with equal volumes of cold sterile saline, and were suspended in cold distilled water.
Preparation of cell walls. Cell walls were prepared by the method of Salton and Horne (15). Washed cells in distilled water were mixed with no. 12 Ballotini glass beads and treated for 30 min in a Mickle shaker. Whole cells and beads were removed by centrifugation at 400 \times g for 20 min. The cell walls were then collected at 12,000 \times g for 30 min in a Servall SS-34 rotor. The upper layer of the sediment was suspended in 0.05 M phosphate buffer (pH 7.0) and again centrifuged at 37,000 \times g for 30 min (17,500 rev/min, Servall SS-34 rotor). After enzyme treatment, the cell walls were washed three additional times with phosphate buffer, dialyzed against distilled water with stirring for 24 hr at 4 C, and then lyophilized. Some cell-wall preparations were further purified by sucrose gradient centrifugation (11).

Preparation of mucopeptide. Mucoprotein was obtained by formamide extraction as described by Krause and McCarty (7). Cell walls were suspended in formamide (2 to 5 mg/ml) and were heated with constant stirring at 170 C for 60 min. The mixture was then cooled to 4 C and centrifuged at 37,000 \times g for 30 min (17,500 rev/min, Servall SS-34 rotor). The supernatant was filtered through Whatman no. 12 paper. The cell walls and beads were removed by centrifugation at 37,000 \times g for 30 min (17,500 rev/min, Servall SS-34 rotor). The sediment was extracted twice to five times with formamide, washed three times with cold water, and dialyzed against distilled water for 24 hr at 4 C. The product on lyophilization was a powder, buff to tan in color. To remove any membrane material which might be contaminating the preparations, some cell-wall suspensions or formamide-derived mucoproteins were extracted for 1% Duponol (sodium lauryl sulfate) and held at 37 C for 4 hr as described by Barkulis et al. (2). These preparations were then washed three times with water and were dialyzed against distilled water in the cold before lyophilization. Preparations were also made from some strains of streptococci by trichloroacetic acid extraction (9) or the warm phenol method (22).

The C. violaceum mucoprotein was obtained by successive extractions of cells with 45 and 88% phenol, followed by acetone extraction and trypsin treatment. The B. cereus preparation was also derived by the phenol extraction followed by pronase digestion. Both of these mucoprotein extracts were obtained from R. W. Wheat.

Preparations were analyzed for rhamnose and nitrogen by the methods previously described (19). Amino acid analysis of the mucoprotein from group A-variant streptococcus, strain B455, was determined with an amino acid analyzer. The molar ratios based on ninhydrin color reaction were: glutamic acid, 1.0; glycine, 0.09; alanine, 2.0; and lysine, 0.8. There were also traces of threonine, valine, and leucine.

The group-specific C polysaccharide was precipitated from the 37,000 \times g formamide supernatant fraction by the method of Fuller (6). Preparation of lysozyme digests. Lysozyme digests were obtained by incubating purified mucoprotein suspensions in 0.05 M phosphate buffer (pH 7.0) with 5 to 50 \mu g of egg white lysozyme per mg of mucoprotein for 2 to 6 hr at 38 C. Change in optical density of the mixture during incubation was followed on a Klett colorimeter with a no. 54 filter.

Animals. New Zealand white rabbits of both sexes, weighing 2,000 to 2,500 g, and random-bred guinea pigs, weighing 600 to 1,200 g, were used.

Immobilization of rabbits. Rabbits were immunized in the footpad with four weekly injections of a saline suspension of 0.5 mg of purified mucoprotein from a group A, strain D-58 streptococcus. An increased concentration of antibody against mucoprotein was observed in all animals (1).

Injection of samples. Mucoprotein, or cell walls, suspended in saline and contained in screw-cap glass bottles, were placed in the cup of a Raytheon 9-kc sonic oscillator with 5 ml of water in the cup. They were subjected to sonic vibration at maximal amplitude for the minimal time necessary to achieve a stable suspension. For most mucoprotein preparations with low polysaccharide content, this was 2 to 10 min. Cell walls were usually treated 10 to 20 min. Animals were injected intradermally in the upper flanks with 100 \mu g in 0.1 ml of sterile saline, unless otherwise specified.

Measurement of skin lesions. The scoring system used in these studies to quantitate the acute lesion was based on the area of involvement in square centimeters, with 1 point for edema, 1 point for redness, and 1 point for necrosis. For example, a reaction of 5 square cm which was edematous, red, and necrotic was scored as 8. The mean score was obtained from a minimum of six animals. A similar method has been utilized by Borel and Schwartz (3) in studies on the development of immediate and delayed hypersensitivity to purified protein antigens in the rabbit. The validity of the method is shown by the direct relationship between mean score and dose in the titration illustrated in Fig. 1. Statistical comparison of means was done by Student's t test.

Microscopic studies. Sections for histological studies were obtained at different time intervals after injection and were fixed in neutral formalin. Paraffin sections were stained with hematoxylin and eosin, or with Giemsa stain.

RESULTS

Tissue reactions to cell walls and cell-wall moieties. Mucoprotein and C polysaccharide were derived by formamide extraction from a group A, strain D58 streptococcus. Approxi-
Table 1. Comparison of acute and chronic skin lesions produced with cell walls and components from group A, D-88 streptococcus

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean index of nodular lesion*</th>
<th>Index per μg peptide injected</th>
<th>Mean acute lesion score</th>
<th>P of mucopeptide vs. cell wall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell wall</td>
<td>260</td>
<td>14</td>
<td>2.0</td>
<td>—</td>
</tr>
<tr>
<td>C polysaccharide</td>
<td>3</td>
<td>0.22</td>
<td>0.9</td>
<td>—</td>
</tr>
<tr>
<td>Mucopeptide</td>
<td>10</td>
<td>0.18</td>
<td>4.9</td>
<td>0.005</td>
</tr>
<tr>
<td>Mucopeptide + C polysaccharide†</td>
<td>18</td>
<td>0.65</td>
<td>4.2</td>
<td>0.005</td>
</tr>
</tbody>
</table>

* Index calculated by dividing maximal area of nodular involvement by time in days for gross appearance of nodules (19).
† Three days after injection.

Equal weights of the two fractions in pH 7.1 phosphate buffer held at 37 C for 2 hr before injection.

Edema and erythema were very apparent within 3 to 4 hr after injection of either cell walls or mucopeptide. There was occasionally some evidence of central necrosis by this time at the mucopeptide site. By 24 to 48 hr, the reactions were more distinct. The cell-wall site subsided and was frequently negative by 3 or 4 days. The mucopeptide site, on the other hand, increased to maximal intensity at about 24 hr, at which time the lesion consisted of an indurated red area up to 25 mm in diameter with an area of central necrosis up to 8 mm in diameter, and elevated 5 to 10 mm. This acute lesion then gradually subsided over a period of 2 weeks, leaving a scar.

Between 3 and 7 days after injection of the intact cell walls, firm, red, nodular lesions appeared at and around the site. Necrosis was not a feature of this late-appearing reaction. This lesion was characteristic of intact cell walls and was only rarely elicited by the mucopeptide fraction. The mixture of C polysaccharide plus mucopeptide did not alter the acute reaction to mucopeptide, nor did it result in an increase in incidence of chronic nodular lesions.

Mucopeptide derived from several other bacterial strains produced acute necrotic reactions essentially similar to that described above. The results of one comparative study are shown in Table 2. Eight normal rabbits were injected at one of six sites with the preparations indicated. Five rabbits, each of which had received two intradermal injections of 200-μg doses of group C streptococcal mucopeptide 1 month apart, were also injected. There was no significant difference between the sensitized and control groups at any of the injection sites.

Another study involving a comparison of mucopeptides from different bacteria is shown in the normal control group in Table 3. As in the previous experiment, the acute necrotic lesions produced by mucopeptides from group A, group A-variant, and group C streptococci, and S. albus were indistinguishable in the gross. The preparations from C. violaceum and B. cereus produced acute, indurated lesions, but with no obvious necrosis.

Nine guinea pigs were injected intradermally with mucopeptide from a group C streptococcus. Acute necrotic lesions, entirely comparable with those described in the rabbits, developed uniformly.

Histological studies. Eight rabbits were injected

Table 2. Comparison at three time intervals of acute lesions produced with mucopeptides from five bacterial strains

<table>
<thead>
<tr>
<th>Source of mucopeptide</th>
<th>Interval after injection (hr)</th>
<th>Normal rabbits (mean score)</th>
<th>Rabbits sensitized with group C, H46A strain* (mean score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group C, strain H46A streptococcus</td>
<td>4</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>6.2</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>2.2</td>
<td>2.9</td>
</tr>
<tr>
<td>Group A, strain D-58 streptococcus</td>
<td>4</td>
<td>5.7</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>7.4</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>4.6</td>
<td>5.3</td>
</tr>
<tr>
<td>Staphylococcus albus</td>
<td>4</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>7.9</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>5.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Chromobacterium violaceum</td>
<td>4</td>
<td>4.4</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>5.8</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>2.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>4</td>
<td>2.6</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>3.3</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>1.3</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* Each rabbit had two previous skin injections of 200 μg of mucopeptide 1 month apart.
Table 3. Comparison of toxicity of mucopeptides from different bacteria in rabbits immunized with group A, D-58 streptococcal mucopeptide and in normal rabbits

<table>
<thead>
<tr>
<th>Bacterial source of mucopeptide</th>
<th>Immunized (mean score)*</th>
<th>Control (mean score)*</th>
<th>P (immune vs. control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A, D-58</td>
<td>5.0</td>
<td>8.2</td>
<td>0.005</td>
</tr>
<tr>
<td>Group A variant, K-43</td>
<td>6.2</td>
<td>9.0</td>
<td>0.01</td>
</tr>
<tr>
<td>Group C, H4A</td>
<td>3.8</td>
<td>7.2</td>
<td>0.005</td>
</tr>
<tr>
<td>Staphylococcus albus</td>
<td>6.3</td>
<td>7.6</td>
<td>0.50</td>
</tr>
<tr>
<td>Chromobacterium violaceum</td>
<td>4.3</td>
<td>7.7</td>
<td>0.025</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>3.9</td>
<td>6.1</td>
<td>0.20</td>
</tr>
</tbody>
</table>

*A time lapse of 24 hr occurred after intradermal injections of 100 μg of mucopeptide.

Fig. 2. Comparison of acute reaction to group C streptococcal cell walls and derived mucopeptide at three intervals after injection. The P value for the difference at 24 hr = 0.005.

on the right anterior flank with 100 μg of cell-wall suspension containing 34% rhamnose obtained from a group C streptococcus. Mucopeptide derived from these walls, containing 4.8% rhamnose, was injected in the midflank. Another mucopeptide preparation from the same strain, but still containing 29% rhamnose, was given posteriorly on the same flank. The reaction against cell walls and mucopeptide is compared in Fig. 2.

At 24 hr after injection, the mucopeptide produced a severe necrotic lesion (Fig. 3A). Figure 3A also illustrates the relationship of acute toxicity to the amount of rhamnose removed. The relatively inactive preparation injected in the posterior site contained a great part of the original cell-wall polysaccharide.

Histological sections from lesions 4 or 24 hr after injection show an intense polymorphonuclear infiltration and consequent tissue disorganization (Fig. 4A). The comparative microscopic studies over the first 24 hr reflect an essentially quantitative difference between cell-wall and mucopeptide sites in infiltration and tissue alteration. Vascular damage and thrombus formation are slight, relative to the extensive cellular reaction.

By 7 days after injection, the mucopeptide-induced lesion had proceeded to heal whereas the anterior site, injected with intact cell walls, had increased in size and was developing the nodular reaction (Fig. 3B). Histological sections taken from the mucopeptide site at this time confirm an essentially healing process (Fig. 4B, C). In contrast, the cell-wall site shows an active chronic inflammatory process with many macrophages and monocytes (Fig. 4D).

Relationship of C polysaccharide to toxicity of mucopeptide. To compare further the relative toxicity of mucopeptides containing varying amounts of residual C polysaccharide, six preparations with rhamnose ranging from 5.5 to 35% were obtained from the same group C streptococcal strain by varying the time of extraction or number of extractions with hot formamide. A comparison of the tissue reaction to these preparations at 4 and 24 hr and at 5 days is shown in Fig. 5. It is seen that the severity of the lesion is directly related to the amount of residual polysaccharide. The most intense reaction was produced by the preparation containing the least amount of polysaccharide (5.5% rhamnose), whereas that containing the highest amount (35% rhamnose) elicited less toxicity and necrosis.

Effect of immunization. Six rabbits were immunized in the footpad with a saline suspension of group A streptococcal mucopeptide. This group and six control rabbits were challenged with intradermal injections of mucopeptides from six bacterial strains (Table 3). The tests for significance of the differences between immunized and control groups indicated that the toxicity of mucopeptides from the three β-hemolytic streptococci was affected to a comparable degree by the immunization. The immunization had no significant effect on reaction to S. albus or B. cereus mucopeptides.

In vitro incubation of group A streptococcal mucopeptide with homologous or heterologous antisera prior to injection failed to demonstrate
Fig. 3. Rabbit injected intradermally with 100 μg of group C streptococcal cell walls in the anterior site (right), 100 μg of mucoprotein containing 4.8% rhamnose in the mid flank, and 100 μg of mucoprotein containing 29% rhamnose in posterior site. (A) 24 hr after injection. (B) 7 days after injection.
Fig. 4. Tissue sections from rabbit skin injected with group C streptococcal cell walls or derived mucopentide. (A) Mucopentide lesion 24 hr after injection. Note intense polymorphonuclear infiltration, disrupted collagen bundles, and relatively clear vessels. Giemsa, X 100. (B) Mucopentide lesion 7 days after injection showing healing with intense fibroblastic activity and no residual inflammatory reaction. Sluffing eschar above with new epithelium beneath and healing proceeding from below. Giemsa, X 100. (C) Same mucopentide lesion as in B at higher magnification to show relative lack of inflammatory cells. Hematoxylin and eosin, X 400. (D) Cell wall lesion from site adjacent to mucopentide lesion in (B) and (C), 7 days after injection. Note extensive acute and chronic inflammation still proceeding, in contrast to mucopentide lesion. Hematoxylin and eosin, X 400.
any neutralization of toxicity. Thus, in spite of the obvious precipitation of mucoprotein with the homologous antiserum A and the cross-reactive antiserum C, injection of these suspensions yielded lesions no different from mucoprotein incubated with anti-B. cereus serum, anti-C. violaceum serum, normal rabbit serum, or saline.

**Effect of lysozyme treatment on toxicity of mucoprotein.** Group A streptococcal mucoprotein (1 mg/ml) was digested with two concentrations of lysozyme (5 and 50 μg/ml). A sample of each of these preparations and a control of mucoprotein in buffer was then centrifuged at 37,000 × g for 30 min. There was negligible sediment in either of the enzyme-treated preparations, whereas all of the mucoprotein control was sedimentable at this speed.

Six rabbits were injected at one of six sites with the following preparations in a concentration equivalent to 100 μg of mucoprotein: each of the enzyme-treated suspensions before centrifugation, the supernatant fractions from each enzyme-treated preparation, mucoprotein control, and a lysozyme control of 50 μg/ml. As shown in Fig. 6, lysozyme reduced the toxicity of mucoprotein. The extent of this reduction was dependent upon the concentration of enzyme. All of the residual toxic effect of lysozyme-treated mucoprotein was in the supernatant fraction, in contrast to the completely sedimentable control suspension.

The effect of lysozyme treatment was even more apparent in a second experiment with a different preparation of group A streptococcal mucoprotein. In this experiment, lysozyme was incubated with mucoprotein in a concentration of 10 μg/mg of substrate for 4 hr at 37 C. By this time, there was no further reduction in turbidity. The suspension was injected without separation of insoluble material by centrifugation. The differences between the lesions produced with this enzyme-treated material and the untreated control mucoprotein were significant at all the time intervals recorded (Table 4).

**Effect of sonic vibration on toxicity of mucoprotein.** A suspension of group A streptococcal mucoprotein was subjected to sonic vibration as described in Materials and Methods. Samples were removed at 2, 10, 30, and 60 min, and each sample was injected into one of four sites on six rabbits. Sonic vibration for a period longer than 10 min significantly reduced the toxicity (Fig. 7).

**DISCUSSION**

Removal of polysaccharide and protein from the mucoprotein structure of cell walls from several bacterial strains renders the mucoprotein capable of producing a severe necrotic reaction in the skin of rabbits and guinea pigs. This lesion is apparent within 3 hr and maximal within 24 hr. Histological studies show that swelling and disruption of the collagen bundles is prominent in these early lesions, but the initial injury is
followed by a healing process with scar formation and no further lesion activity.

Similar microscopic alterations of the connective tissue elements are observed in the early reaction to intact cell walls (5). The lesions in this case are quantitatively less intense than those obtained with the derived mucopептид. The distinguishing feature of the reaction after cell-wall injection is the sustained irritation, yielding a chronic granulomatous process which becomes macroscopically apparent as a nodular lesion after a latent period of several days.

On the basis of these observations, it is proposed that the toxic moiety of cell walls from β-hemolytic streptococci and probably most gram-positive bacteria is an integral part of the mucopептид structure. The associated polysaccharide, and perhaps the protease-resistant proteins, masks these reactive sites, slowing the reaction with tissue. Free mucopептид is readily lysed by lysozyme in contrast to the resistance of cell walls from most bacteria. This implies that the polysaccharide also functions to protect the mucopептид from tissue muramidases. Thus, mucopептид complexed with polysaccharide can persist in tissue, producing the chronic inflammation characterized grossly by remittent nodules.

In a previous paper, we observed several properties of bacterial cells, both intrinsic and extrinsic to the cell wall, which help explain the variation between bacterial strains in the capacity to elicit the chronic nodular lesion (17). Variation of those structural groups on mucopептид involved in the reaction with tissue and the relationship of such groups to an associated polysaccharide are further sources of the qualitative and quantitative differences in reactions to cell-wall fragments from different bacteria.

The mechanism by which mucopептид produces the acute tissue reaction is unknown. The fact that nearly all normal rabbit sera have precipitating antibody to mucopептид (1) might suggest that the basis for the reaction is hypersensitivity. This may be a contributing factor to the acute inflammation, as indicated by previous studies on the role of hypersensitivity, in the early phases of the reaction to intact cell walls (16). However, two or three repeated skin-test injections of mucopептид did not enhance the acute reaction, whereas hyperimmunization by the footpad route significantly reduced it. The induration, elevation, and lack of severe hemorrhage, as well as the moderate vascular involvement relative to the extensive tissue necrosis, further serve to distinguish this reaction from antibody-mediated lesions such as the Arthus reaction.

The possibility that the acute toxic activity is an artifact of the drastic methods used to obtain mucopептиды must be considered. Three observations argue against this explanation: (i) antiserum from animals immunized with untreated whole cells or cell walls can react specifically with the derived mucopептид, indicating that antigenic structure remains intact (1; Abdulla and Schwab, Federation Proc. 24: 186, 1965); (ii) the acute reaction over the first 24 hr is quantitatively, rather than qualitatively, different from that obtained with untreated cell walls; (iii) extraction of cell walls with trichloroacetic acid or phenol yields products having essentially the same relative toxicity as those derived by formaldehyde treatment.

Evidence that the preparations are homogeneous, and that toxicity is attributable directly to the mucopептид, includes the following: amino acid analysis of the material shows those amino acids and amino sugars characteristic of cell-wall mucopептид (14); there is negligible absorbance of ultraviolet light from 320 to 240 μν; treatment of mucopептид with ribonuclease, pronase, papain, or trypsin does not affect toxicity; extraction with Duponol, trichloroacetic acid, or phenol leaves the toxicity unaffected; and, finally, lysozyme digestion reduces toxicity.
It was previously reported that streptococcal cell walls, carefully prepared to prevent contamination with endotoxin from gram-negative bacteria, had about 2 $\times$ 10$^{-3}$ of the pyrogenic and other pharmacological effects of endotoxin (12). From these quantitative considerations, and published reports on skin reactions to endotoxins (8, 21), it is concluded that the reaction to mucopeptide is quite distinct from that elicited by endotoxins. However, mucopeptide could participate in the tissue injury produced with intact cell walls from gram-negative organisms.

The particle size of the mucopeptide is an important factor in obtaining the necrotic lesions described here. The optimal period of sonic vibration for resuspension of the mucopeptides varies somewhat between preparations. Prolonged sonic vibration significantly reduces toxic activity. It has been emphasized previously that the nature of tissue response can be greatly influenced by the size of injected particles (10, 12, 13).

The specificity of the resistance to toxicity in immunized animals is evident from the results of challenge with homologous and heterologous preparations. The degree of protection against some of the heterologous mucopeptides is consistent with observations on antigenic groups shared by these preparations (Abdulla and Schwab, Federation Proc. 24:186, 1965). The failure of precipitating antibodies to neutralize biological activity when mixed in vitro is not unusual (4). It suggests that the resistance demonstrated by challenge of immunized animals may not involve circulating antibodies, or that a much higher ratio of antibody to antigen is needed than was obtained in the test tube.

ACKNOWLEDGMENTS

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LITERATURE CITED


