Cell Wall Component Which Affects the Ability of Serum to Promote Phagocytosis and Killing of *Staphylococcus aureus*

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The role of various cell wall components of nonencapsulated *Staphylococcus aureus* in phagocytosis and killing was studied. Adsorption of human serum with *S. aureus* cell walls, or trypsin-digested cell walls, reduced the rate of phagocytosis and killing of *S. aureus* by human-blood leukocytes when compared to unadsorbed serum. When the various components of the cell walls were used for adsorption, only the intact muropeptide was found to remove these factors. Products of digestion of this muropeptide with several enzymes were found to be inactive when used for adsorption of human serum. Other components tested were teichoic acids α, β, and combinations of both and protein A. When used in equivalent amounts, these were found to have no effect on the removal of serum factors necessary for phagocytosis and intracellular killing of *S. aureus*.

It has been shown in the previous report (19) that *Staphylococcus aureus* strains, as well as some other gram-positive and gram-negative bacteria, yeast, and some inert particles, adsorbed the phagocytosis-promoting factors for *S. aureus* from human sera. The following studies were undertaken to determine which of the available cell wall components of nonencapsulated *S. aureus* of the ordinary kind are involved in the interaction of sera, bacteria, and leukocytes.

The *S. aureus* cell wall preparation, which removed phagocytosis-promoting and killing factors from serum, was further refined to obtain muropeptide, muropeptide digests, teichoic acids, and protein A. When these components were used for adsorption of serum, only the muropeptide was found to be involved in the interaction.

As it was previously reported (15) that adsorption with *S. aureus* cell wall teichoic acid removed the phagocytosis-promoting and killing action for *S. aureus*, this study has been reviewed quantitatively in the present report, by using the teichoic acids from several strains of *S. aureus*.

Immune rabbit sera were absorbed with homologous cell wall teichoic acids or protein A at the equivalence point as tested in double-diffusion agar plates. The absorbed and unabsorbed sera had the same effect on the rate of phagocytosis and intracellular killing of homologous *S. aureus* in normal rabbit polymorphonuclear leukocytes or monocytes. Similar results were obtained when normal human serum was absorbed with teichoic acid and protein A and tested with human-blood leukocytes. In view of the present report, it can be concluded that the interpretation (15) that the teichoic acids are critical acceptors for phagocytosis-promoting antibodies was in error, probably due to the great excess of teichoic acid used for absorption.

**MATERIALS AND METHODS**

*S. aureus* strains used in these experiments, the procedure for immunization of rabbits with heat-killed *S. aureus*, the agar-gel precipitation test, preparation of human-blood leukocytes, rabbit exudative monocytes, and polymorphonuclear leukocytes, and the experimental procedures are the same as described in the previous study (19).

Preparation of *S. aureus* cell wall components: undigested cell walls of *S. aureus* 3528 and Micrococcus lysodeikticus. The bacteria were cultured at 38°C for 20 hr with aeration in a medium consisting of beef extract, 5 g; glucose, 10 g; NaCl, 1 g; Na₂HPO₄·2H₂O, 7.5 g; NaH₂PO₄, 3 g; peptone, 10 g; NH₄Cl, 1 g; Na₂SO₄·10H₂O, 0.5 g; MgSO₄·7H₂O, 6.2 g; thiamine, 2 mg; and nicotinic acid, 4 mg/liter of distilled water.

Disintegration of the washed cells essentially

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followed the method of Bleiweis et al. (2) by using a Britton homogenizer with glass powder. The disintegrated cell suspension, after filtration through a glass filter to remove the glass powder, was centrifuged at 4,000 rev/min (Sorvall SS-34 Rotor) for 10 min. The supernatant was again centrifuged at 10,500 rev/min for 30 min to obtain the sediment containing "crude cell walls." The crude cell walls were washed twice each with 1 M NaCl and distilled water and then lyophilized to obtain the "undigested cell walls." Approximately 1.5 g (dry weight) or S. aureus was used for disintegration, and 280 mg (dry) of undigested cell walls was yielded.

Digested cell walls of S. aureus 3528: The undigested cell walls (240 mg) were digested twice with 10 mg of crystalline trypsin (Type III, Sigma Chemical Co., St. Louis, Mo.) in 1/15 M phosphate buffer (pH 7.8) at 38°C for 1 hr each time. The digested cell walls were washed twice each with the same buffer and distilled water and were then lyophilized to get 192 mg of the digested cell walls.

**S. aureus 3528 cell wall mucoprotein (pepido-glycan or murein).** A 3.2-g amount of the digested cell walls was extracted with cold 10% trichloroacetic acid at 0°C for 3 days by the method of Baddiley et al. (1), followed by centrifugation. The sediment, after extensive washing with alcohol and distilled water and lyophilizing, was further extracted with 10% trichloroacetic acid at 60°C for 2 hr and centrifuged at 10,500 rev/min for 30 min. The sediment was washed with alcohol twice and distilled water three times; it was then lyophilized to obtain 1.7 g of the staphylococcal mucoprotein.

**Digestion of staphylococcal mucoprotein with egg-white lysozyme.** A 400-mg amount of staphylococcal mucoprotein was suspended in 40 ml of 0.05 M ammonium acetate buffer (pH 6.5) containing 8 mg of egg-white lysozyme (Sigma Chemical Co.). After incubation for 24 hr at 37°C, 8 ml of cold 60% trichloroacetic acid was added to the digest, and the mixture was allowed to stand for 30 min in the ice bath. The resultant precipitate was removed by centrifugation, and the supernatant was dialyzed against cold running water for 24 hr and cold distilled water for 24 hr. The nondiffusible fraction was lyophilized to yield 280 mg of a soluble white powder.

**Digestion of staphylococcal mucoprotein with myxobacter AL-1 protease.** The staphylococcal mucoprotein (690 mg) was suspended in 20 ml of 0.02 M sodium barbital buffer (pH 8.9) containing 3.5 mg of myxobacter AL-1 protease (18,500 units/mg) and incubated at 37°C for 24 hr. (The enzyme (5, 20) was kindly provided by R. S. Wolfe, Department of Microbiology, University of Illinois, Urbana.) The pH of the digest was kept at 8.9 by addition of 1 N NaOH. After digestion was complete, the digest was treated in the same way as the lysozyme digest of staphylococcal mucoprotein to yield 259 mg of soluble white powder.

**Digestion of staphylococcal mucoprotein with staphylococcal endo-β-N-acetyl-glucosaminidase.** Staphylococcal mucoprotein (600 mg) was suspended in 44 ml of 0.05 M ammonium acetate buffer (pH 6.5) containing 2,400 units of staphylococcal endo-β-N-acetyl-glucosaminidase (T. Wadström and K. Hisatsume, Biochem J. 120:735-744) and several drops of toluene and incubated at 37°C for 18 hr. The digest was dialyzed against 150 ml of distilled water at 2°C for 24 hr. Dialysis was repeated three times, and the dialyzable fraction was collected by lyophilization. The white powder obtained after lyophilization was dissolved in a small amount of distilled water and evaporated in vacuo in the presence of KOH to remove ammonium acetate from the material. This procedure was repeated three times to yield 405 mg of the material.

**Preparation of S. aureus cell wall teichoic acid.** The method of Baddiley et al. (1) was used for preparation of teichoic acid from S. aureus strains. The Copenhagen strain teichoic acid-glycopeptide complex was kindly provided by Jack L. Strominger.

**Preparation of protein A from S. aureus cell walls.** Protein A from cell walls of S. aureus was prepared by the method of Forsgren and Sjöquist (7), in which crude protein A was partially purified by chromatography on diethylaminoethyl (DEAE) and G-100 Sephadex.

Protein A also was kindly supplied by John Sjöquist of the University of Uppsala, Sweden. This was prepared in the following manner. The cells were digested by lysoctaphin. The supernatant was adjusted to pH 3.5, by addition of HCl, and the precipitate formed was centrifuged off and discarded. The supernatant was neutralized and ammonium sulfate was added to 80% saturation. The precipitate was recovered by centrifugation, dissolved in distilled water, and dialyzed against 0.1 M ammonium bicarbonate. Chromatography was done repeatedly on DEAE and G-150 Sephadex (*personal communication*).

**Adsorption of serum.** To 1 ml of 1:2 diluted serum was added the S. aureus cell wall component in the amount stated in experimental results. The mixture was rotated at 37°C for 60 min and kept in the refrigerator overnight. It was then centrifuged and the supernatant was filtered through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.). A sample of serum which did not contain adsorbing materials was processed in an identical manner and used as control.

**RESULTS**

Adsorption of normal human serum with S. aureus cell walls and mucoprotein. S. aureus 3528 cell walls, undigested or digested with trypsin, and cell wall mucoprotein, prepared as described above, were used for adsorption of normal human serum. A 2-mg amount of these cell wall components, the amount comparable to 2 × 10^11 organisms, was used for adsorption of 1 ml of 1:2 diluted normal human serum as mentioned above. Figure 1 represents the mean values of experiments on autologous blood leukocytes and serum for six normal humans by using the method for viable counts and S. aureus 3528 as test organism. The cell-bound curves show that with unadsorbed serum there is phagocytosis for the
first 30 min followed by phagocytosis and reduction in viable count. With adsorbed sera, however, the phagocytosis was slow with little killing. In the total viable count, the rate of killing with adsorbed sera also was much lower than with unadsorbed serum ($P < 0.005$).

Adsortion of normal human serum with *M. lysodeikticus* cell walls. Cell walls of *Micrococcus lysodeikticus*, which contain a great quantity of mucopeptide, were prepared and used for adsorption of human serum. Figure 2 shows the mean values of experiments on autologous blood leukocytes and serum of three normal humans. Adsorption of 1 ml of 1:2 diluted serum with 2 mg of *M. lysodeikticus* cell walls reduced the phagocytosis and intracellular killing of *S. aureus* 3528 ($P < 0.005$) to the same extent as did the homologous mucopeptide.

Absorption of normal serum with digests of the cell wall mucopeptide. The cell wall mucopeptide of *S. aureus* contains a carbohydrate backbone of alternate molecules of N-acetylmuramic acid and N-acetylglucosamine; the cell wall peptide is attached to the carboxylic groups of the muramic acid through peptide linkages. The tetrapeptide side chains are cross linked by pentaglycine bridges. Structural formulae have been reproduced (14).

Mucopeptide of *S. aureus* 3528 was split by the use of three different lytic enzymes.

(i) Egg-white lysozyme [3 (endo-β-N-acetyl-

muramidase)] breaks the polysaccharide backbone between N-acetyl-muramic acid and N-acetylglucosamine, leaving the peptide intact.

(ii) Myxobacter L-1 protease (20) solubilizes the cell walls of *S. aureus* by a primary attack on the pentaglycine cross-bridges which interlink peptidoglycan strands. Hydrolysis of the N-acetyl-muramyl-L-alanine linkages between the polysaccharide and the peptides occurs with eventual liberation of small peptide fragments, undegraded polysaccharide, and a polysaccharide-teichoic acid complex.


Individual samples of 1 ml of normal human serum (1:2 dilution) were absorbed with 1 or 2 mg of each of these soluble digests or with a combination of the lysozyme and protease digests (1 mg each). Figure 3 represents the mean values of
experiments on autologous blood leukocytes and sera from two to seven normal humans. It can be seen that absorption with the three soluble mucoprotein digests alone or in the above combination did not have any significant effect (P > 0.25) on the ability of the serum to promote phagocytosis and killing in contrast to adsorption with the undigested, intact mucoprotein.

Absorption of immune rabbit serum and normal human serum with S. aureus cell wall teichoic acids.

It was reported (15) that absorption of human serum with S. aureus cell wall teichoic acid removed both the phagocytosis-promoting and killing effects of serum. In that report an excessively large amount of teichoic acid was used for absorption (25 mg/ml of 1:10 serum). Since this large amount of excess soluble antigen remained in the test system, it might have had an inhibitory effect on the leukocyte functions.

In the present study, rabbits were immunized with the following heat-killed S. aureus strains: (i) 3528, which contains 95 to 100% α and 0 to 5% β teichoic acid; (ii) H, which contains 0 to 5% α and 95 to 100% β teichoic acid (16); and (iii) Copenhagen, 18Z, and Wood 46, which contain a combination of both. The antisera were absorbed with teichoic acid from the homologous organism and tested against the homologous organism. The amount of teichoic acid used for absorption was based on the determination of the equivalence point of antigen-antibody reaction. An example, Fig. 4, shows a diagram of precipitin lines on an Ouchterlony plate with rabbit immune serum absorbed with various amounts of the homologous teichoic acid, from 10 µg/ml of one half diluted serum to 500 µg. Absorption with 500 µg of soluble teichoic acid was in the area of excess antigen, giving a heavy line with the neighboring wells of unabsorbed serum or serum absorbed with 25 µg/ml of teichoic acid but showing no reaction with the center well containing 10 µg of teichoic acid. Sera, either unabsorbed or absorbed with 10, 25, and 50 µg of teichoic acid show precipitin lines with the antigen in the center well. As 100 µg was found to be the equivalence point for this and other teichoic acid-anti-teichoic acid reactions, this was the amount used for all absorptions.

Figure 5A shows an example of an experiment in which normal rabbit monocytes and rabbit anti-Wood 46 serum were absorbed with 100 µg of Wood 46 teichoic acid, the equivalence point, or with 500 µg, an excess of antigen. Results from these two sera are not significantly different from that of the unabsorbed serum. The same result was observed (Fig. 5B) when normal human serum, which contains some anti-teichoic acid antibody, was absorbed with 100 µg of teichoic acids from 3528, H, and 18Z strains, the homologous S. aureus strain being used in the test, and compared with their corresponding unabsorbed sera.

Table 1 shows the statistical analyses of results of absorption of sera with teichoic acids in a variety of conditions. Rabbits were immunized with different strains of heat-killed S. aureus. The immune sera were then absorbed with 100 µg of the homologous teichoic acids and were used in the test with either normal rabbit polymorphonuclear leukocytes or monocytes and the homologous organisms. Normal human sera, absorbed

**Fig. 4.** Determination of the equivalence point is shown by precipitin lines by using 10 µg of S. aureus 18Z cell wall teichoic acid (labeled T/A) and rabbit S. aureus 18Z antisera absorbed with various amounts of the same teichoic acid (labeled as numbers of micrograms used for absorption).

**Fig. 5.** Effect of absorption of serum with teichoic acid on the intracellular fate of S. aureus is compared to the same serum unabsorbed. (a) Rabbit anti-Wood 46 serum absorbed with S. aureus Wood 46 teichoic acid and tested with the same organism in normal rabbit monocytes. (b) Normal human serum absorbed with teichoic acids from various strains and tested with the same organism in normal human blood leukocytes.
TABLE 1. Statistical analyses of variance comparing the effect of sera absorbed with Staphylococcus aureus cell wall teichoic acid with that of the same sera unabsorbed

<table>
<thead>
<tr>
<th>Leukocytes</th>
<th>Rabbit antiserum against S. aureus strains</th>
<th>Absorbed with S. aureus cell wall teichoic acids</th>
<th>S. aureus used in the test</th>
<th>No. of tests</th>
<th>Statistical analyses of variance between absorbed and unabsorbed sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rabbit peritoneal PMN leukocytes</td>
<td>3528, H, Wood 46, 18Z, Copenhagen</td>
<td>3528, H, Wood 46, 18Z, Copenhagen</td>
<td>3528, H, Wood 46, 18Z, Copenhagen</td>
<td>9</td>
<td>$P &gt; 0.5$</td>
</tr>
<tr>
<td>Normal rabbit peritoneal monocytes</td>
<td>3528, H, Wood 46, 18Z, Copenhagen</td>
<td>3528, H, Wood 46, 18Z, Copenhagen</td>
<td>3528, H, Wood 46, 18Z, Copenhagen</td>
<td>10</td>
<td>$P &gt; 0.5$</td>
</tr>
<tr>
<td>Normal human blood leukocytes</td>
<td>Normal human serum</td>
<td>3528, H, Wood 46, 18Z</td>
<td>3528, H, Wood 46, 18Z</td>
<td>14</td>
<td>$P &gt; 0.5$, $P &gt; 0.25$</td>
</tr>
</tbody>
</table>

with 100 μg of the different teichoic acids, were used with human blood leukocytes, and the strain of *S. aureus* corresponding to each of the teichoic acids was used for absorption. Experimental results were obtained by viable counts of intracellular and total bacteria and by microscopic examination of stained cover slips for the number of leukocytes containing bacteria. Statistical analyses of variances of results from this variety of conditions show that differences between use of absorbed and unabsorbed sera are well below the amount conventionally regarded as significant in all cases.

The contradiction between results given here and that of previous work (15) could be due to the extremely large antigen excess formerly used, resulting in nonspecific effects.

Absorption of serum to determine the relationship of *S. aureus* cell wall protein A to phagocytosis and killing. Three different methods were used to test the effect of protein A on phagocytosis and killing of *S. aureus*. As has already been shown in Fig. 1, digested cell walls, which lack protein A, behaved similarly to the undigested cell walls, in that the factors necessary for phagocytosis and killing were removed to about the same extent. The second method compares the effect of adsorption of human serum with heat-killed and live *S. aureus* 46, which lacks protein A, to that of adsorption with Cowan I, which contains a large amount of protein A. Procedures for adsorption have been described in the previous report (19).

Figure 6 shows that adsorption with either of these organisms removed the phagocytosis- and killing-promoting factors for *S. aureus* 18Z, again to the same extent. The direct effect of protein A was studied by using a preparation of protein A obtained from *S. aureus* 18Z. The sera from rabbits immunized with heat-killed *S. aureus* 18Z were absorbed with various amounts of protein A and were tested in Ouchterlony plates for determination of the equivalence point of antigen-antibody. Figure 7A shows the results of such an experiment with normal rabbit monocytes and rabbit anti-*S. aureus* 18Z serum absorbed with 50 μg of *S. aureus* 18Z protein A (equivalence point). In Fig. 7B, human sera was absorbed with an amount equal to that used with the rabbit antiserum and tested with autologous human blood leukocytes. In both cases *S. aureus* 18Z was used as test organism. No significant difference was noted in either case when compared to unabsorbed serum ($P > 0.5$).

A preparation of protein A supplied by John Sjöquist was also used for absorption of serum from a rabbit immunized by repeated infection with live *S. aureus* 18Z and phage-lysed *staphylococci*. Immune serum was absorbed with different concentrations of protein A and the equivalence point (100 μg of protein A/ml of 1:2 diluted serum) was determined by Ouchterlony plates. Again, absorbed and unabsorbed sera behaved similarly in phagocytosis and intracellular killing of *S. aureus* 18Z.
teichoic acids are  N-acetyl-glucosaminides of ribitol phosphate with D-alanine ester linkage and occur in two forms, depending upon whether the  N-acetyl-glucosaminide is linked in  \( \alpha \) or  \( \beta \) configuration. The surface of  \( S. aureus \) also contains various factor-specific antigens and phage receptor sites. These materials occur in very small amounts and have not been isolated from the cell wall materials (14).

In the present study, attempts have been made to identify which one of the available cell wall components is most directly involved in the interaction between serum, bacteria, and leukocytes.

Mudd et al. (15) reported that the phagocytosis-promoting factors and killing of fresh human serum could be removed by adsorption with  \( S. aureus \) cell wall teichoic acid or with  \( \alpha, \beta \)-methyl-\( N \)-acetyl-d-glucosaminide. However, large amounts of absorbing materials were used, and the presence of an excessive amount of soluble antigen in the test system might have had a nonspecific effect on phagocytosis. In the present study the effect of absorption of hyperimmune rabbit sera with homologous  \( S. aureus \) cell wall teichoic acids of  \( \alpha, \beta \) or  \( \alpha-\beta \) configurations has been quantitatively reviewed. Serum absorbed with teichoic acid at the equivalence point of antigen-antibody, as tested in Ouchterlony plates, behaved similarly to the unabsorbed serum when used for phagocytosis and intracellular fate of  \( S. aureus \).

Forsgren and Sjöquist (7, 8) and Forsgren (6) reported that protein A from  \( S. aureus \) reacts nonspecifically with the Fc fragment of gamma-G globulin. Martin and White (13) indicated the presence of both specific and nonspecific antigen A precipitins in human sera. Lind and Mansa (10) reported that some strains of  \( S. aureus \), termed reactive, absorbed all gamma-G globulins including homologous antibodies, whereas others, which they termed nonreactive, absorbed only the homologous antibodies. They feel that the reactive strains might contain a large amount of protein A. Dossett et al. (4) reported that staphylococcal protein A has an antiphagocytic effect. The amount of protein A in the systems was not stated. In our study we have found that protein A in equivalent amounts had no effect on phagocytosis and killing. This has been shown by three different methods involving the adsorption of serum. The absorbing materials used for comparison were (i) undigested cell walls and cell walls in which protein has been removed by digestion, (ii) Wood 46 which contains no detectable protein A and Cowan I which contains a large amount of protein A, and (iii) extracted soluble protein A, the amount used being determined by the equivalence point on Ouchterlony plates. Since an

**DISCUSSION**

The cell wall of nonencapsulated  \( S. aureus \) includes an outer layer usually containing protein A and an inner layer of a mucoprotein-teichoic acid complex which probably also is exposed to some degree at the surface. The

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**Figure 6.** Total viable counts of  \( S. aureus \) 18Z compared the effect of adsorption of human serum with  \( S. aureus \) Cowan I (containing protein A) on the fate of the bacteria with that of the same serum adsorbed with  \( S. aureus \) Wood 46 (lacking protein A), by using autologous human blood leukocytes.

**Figure 7.** Effect of absorption with  \( S. aureus \) 18Z protein A on the intracellular fate of  \( S. aureus \) 18Z is shown (A) in rabbit  \( S. aureus \) 18Z antiserum and normal rabbit monocytes and (B) human serum and blood leukocytes.
equivalent amount of antigen is used for absorption, this eliminates the possibility that any excess antigen might have acted directly on the leukocytes to impair their functions in a relative and undetectable way. In the first two comparisons, phagocytosis and killing were reduced equally, whereas in the third comparison phagocytosis and killing were the same with either absorbed or unabsorbed serum.

Adsorption of human serum with *S. aureus* cell walls, undigested or digested with trypsin, and the cell wall murcopeptide significantly reduced the phagocytosis-promoting factors for *S. aureus* as did cell walls of *M. lysodeikticus*. The products of degradation of the murcopeptide with various enzymes, when used for adsorption, were without effect.

The previous report (19) showed that adsorption of serum with *S. aureus*, *S. albus*, *B. subtilis*, *E. coli*, yeast, and inert particles such as polystyrene, carbon, silica, bentonite, but not small glass beads, partially or completely removed phagocytosis-promoting factors. In the present report, the insoluble products of *S. aureus* such as the cell wall and cell wall mucopeptide, when used for adsorption of serum, were effective in reducing phagocytosis and killing, but soluble cell wall products such as mucopeptide digests, teichoic acid, and protein A were ineffective. The efficacy of cell walls of *M. lysodeikticus* in adsorption raises the interesting question as to how general the interaction of murcopeptide with serum opsonins may be. At present, we do not have clear evidence concerning the specificity of this interaction.

The failure to augment the phagocytic activity of normal leukocytes with antibacterial serum from immunized animals has also been shown in the previous report (19). However, since human polymorphonuclear leukocytes and macrophages are both susceptible to injury by staphylococcal leukocidin, and human macrophages are susceptible to α toxin, antitoxin against staphylococcal toxins are protective of the phagocytic function (9, 14). Augmentation of resistance through activation of reticuloendothelial function has been shown with some organisms including *Mycobacterium tuberculosis* (11), Brucella (18), Listeria (12), and Salmonella (17). The possibility of cell-mediated resistance in relation to *S. aureus* is being studied by using other methods of immunization.

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**LITERATURE CITED**