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Cryptic Peptidoglycan and the Antiphagocytic Effect of the *Staphylococcus aureus* Capsule: Model for the Antiphagocytic Effect of Bacterial Cell Surface Polymers

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The antiphagocytic effect of the *Staphylococcus aureus* capsule is known to be related to its ability to interfere with opsonization by normal human serum. In this study, evidence is presented with isolated cell surface components which indicates that the capsule hinders opsonization by masking cell wall peptidoglycan. In contrast to intact, encapsulated *S. aureus* M cells, peptidoglycan particles isolated from the organism were efficiently opsonized by normal human serum and phagocytized by human polymorphonuclear leukocytes. Cell wall particles retaining capsular material were opsonized less efficiently than peptidoglycan. Studies comparing the opsonic capacities of normal, C2-deficient, and heat-inactivated sera led to the conclusion that both the classical and the alternative complement pathways contribute to the opsonization of peptidoglycan in normal human serum. It appears that the capsule interferes with opsonization via both of these complement pathways. Serum from rabbits immunized with *S. aureus* M had significant heat-stable opsonic activity for the intact organism and cell walls retaining capsular material, but not for peptidoglycan. A general model is proposed to explain how antiphagocytic cell surface polymers may inhibit bacterial opsonization and thereby impede natural immunity.

The important role of phagocytosis in host defense against invading bacterial pathogens is well demonstrated by the increased susceptibility to infectious disease manifest in patients with a wide variety of abnormalities of phagocyte function (23). For normal phagocytic cells to ingest and perform their bactericidal function, they must first be able to recognize the bacterium. Recognition depends on the process of opsonization by serum factors. These serum factors (opsonins) were first defined by Wright and Douglas (29) in 1903 as "elements in blood fluids which modify bacteria rendering them a ready prey to the phagocytes." Certain bacteria are able to evade the normal phagocytic defense mechanism, and this "resistance to phagocytosis" often appears to be related to production of a capsule by such organisms.

In the case of unencapsulated *Staphylococcus aureus* strains, phagocytosis is promoted both by heat-labile factors of the serum complement system (10) and by heat-stable factors, primarily immunoglobulin G (15), both of which are present in sera obtained from normal donors without a history of staphylococcal disease. There thus exists in this sense "natural immunity" to *S. aureus*. Results of recent studies led us to conclude that peptidoglycan (PG) exposed at the staphylococcal cell surface plays a key role in promoting opsonization of this bacterial species, since isolated PG, purified cell walls (PG plus covalently linked teichoic acid), and intact bacteria showed very similar opsonic requirements for phagocytosis (18). Also, a teichoic acid-deficient *S. aureus* mutant was opsonized and phagocytized very similarly to its teichoic acid-containing parent (18). These observations suggest that teichoic acid is neither required for staphylococcal opsonization, nor does it impede opsonization.

The presence of a capsule is a convincing virulence factor in strains of several different bacterial species (1, 3, 5, 12, 13, 16, 19, 22, 28) which correlates well with the ability of encapsulated strains to resist phagocytosis (12, 22). Capsular material is a cell surface component which is located external to the cell wall and is usually polysaccharide in nature. These antigenic capsules form part of the effective surface of the organisms, and type-specific acquired immunity can be ascribed to the development of antibodies directed against these polymers.

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In recent studies with a heavily encapsulated strain, *S. aureus* M, we found this organism to be ineffectively opsonized by the classical and the alternative complement pathways and by heat-stable factors in normal human serum (19). This conclusion was reached by separating the processes of opsonization and phagocytosis through incubation of the particle under study with the putative opsonic source (serum) before presenting the preincubated particles to the phagocytes. Since the encapsulated M strain was not phagocytized after incubation in sera opsonic for its unencapsulated variant, it was concluded that the M strain had not become opsonized according to the definition of Wright and Douglas (29); i.e., significantly fewer encapsulated organisms were phagocytized after incubation in normal serum.

In this communication we present evidence, using the M strain, which supports the hypothesis that the antiphagocytic property of the capsule of this organism is related to its masking of cell wall PG. We also suggest that this hypothesis may serve as a model to explain the antiphagocytic effects of cell surface polymers in other microbial species.

MATERIALS AND METHODS

Strains, cultural conditions, and radioactive labeling. *S. aureus* M and an unencapsulated variant of this organism were kindly provided by M. A. Melly, Vanderbilt School of Medicine, Nashville, Tenn. (13, 19). Routinely, the strains were grown in a broth composed of 5 g of phytone peptone (Baltimore Biological Laboratory, Cockeysville, Md.), 5 g of yeast extract (Difco Laboratories, Detroit, Mich.), 2 g of glucose, and 3 g of K_2HPO_4 per liter at pH 7.4, and 37°C with shaking for 18 h (18). For use in phagocytosis experiments, organisms were labeled by growing in 20 ml of broth containing 0.04 mCi of [2-^{3}H]glycine (specific activity, 5 to 15 Ci/mmol; New England Nuclear Corp., Boston, Mass). Cells which were to be used for preparing labeled cell walls retaining capsular material (CW+C) and PG were grown in broth containing 0.4 μCi of [2-^{3}H]glycine per ml. For isolation of capsular polysaccharide as a source of marker compounds for the amino acid analyzer, *S. aureus* M was grown in brain heart infusion broth (Difco) as described by Liau et al. (11).

Preparation of CW+C and PG and their chemical characterization. CW+C were isolated from 1 to 2 liters of labeled M strain by a cell wall isolation procedure modified to aid in retaining capsular material (18). Organisms were not washed after harvesting, and, after breakage, crude CW+C were resuspended in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.5, containing 2% (wt/vol) sodium dodecyl sulfate and stirred for 1 h at 22°C. Next, the crude CW+C were washed twice with water and three times with 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.5. Pellets were sloppy, presumably due to the presence of capsular material, and centrifugation was increased to 39,000 × *g* for 5 min to yield firmer pellets. Nuclease and trypsin treatments were as described previously (18); phenol extraction was omitted; the CW+C were finally washed five times in water. PG was prepared by treating CW+C in 10% (wt/vol) trichloroacetic acid at 60°C for 90 min (18). This acid treatment removes both capsular material and teichoic acid and yielded a firm pellet. The preparations were characterized chemically by amino acid analysis after hydrolysis in 6 M HCl at 105°C for 18 h, and total phosphorus was estimated colorimetrically (18).

Isolation of capsular polysaccharide. Capsular polysaccharide was extracted from brain heart infusion broth-grown M strain by hot, dilute acid treatment followed by enzymatic digestion and Sephadex G-100 chromatography, as described by Liau et al. (11).

Opsonic sources. Serum collected from four healthy donors, who denied previous staphylococcal infection, was pooled and used as a source of normal serum. To study opsonization via the alternative pathway, serum was obtained from a donor with a genetically determined complete and selective deficiency of C2 (6). *S. aureus* M immune rabbit serum was obtained from a rabbit immunized with heat-killed *S. aureus* M (19). All serum sources were stored in 0.5-ml portions at −70°C and thawed just before use. Complement was inactivated by heating serum at 56°C for 30 min. Serum was diluted to a 10% concentration in Hanks balanced salt solution containing 0.1% (wt/vol) gelatin.

Opsonization procedure. By a previously described method (19), particles were incubated in the indicated opsonic sources for specified times and were then centrifuged and resuspended in Hanks balanced salt solution before being presented to polymorphonuclear leukocytes (PMNs).

Preparation of PMNs. Human PMNs were isolated from healthy donors as described previously (19).

Measurement of phagocytosis. Phagocytosis was determined by a previously described method (19), and results were expressed as a percentage of the total radioactivity taken up by PMNs. When intact bacteria were used, 4×10^7 colony-forming units were presented to 10^6 PMNs, yielding a ratio of bacteria to PMN of 40:1. Since 4×10^7 organisms yield approximately 10 μg of *S. aureus* cell walls (18), 10 μg of CW+C and 10 μg of PG were presented to 10^6 PMNs.

Electron microscopy. Intact bacteria, CW+C, and PG were incubated for 1 h in 20% *S. aureus* M immune rabbit serum to demonstrate capsular material (13). Samples were fixed with 2% (wt/vol) glutaraldehyde in cacodylate buffer; buffered OsO_4 was added, and staining was en bloc with 1% (wt/vol) uranyl acetate. Thin sections were examined in a Siemens Elmiskop 1 electron microscope.

RESULTS

Characterization of the CW+C and PG preparations. In Fig. 1, electron micrographs of thin sections of intact bacteria, CW+C, and PG are shown. To demonstrate the presence or the absence of capsular material, these preparations had been previously incubated in serum

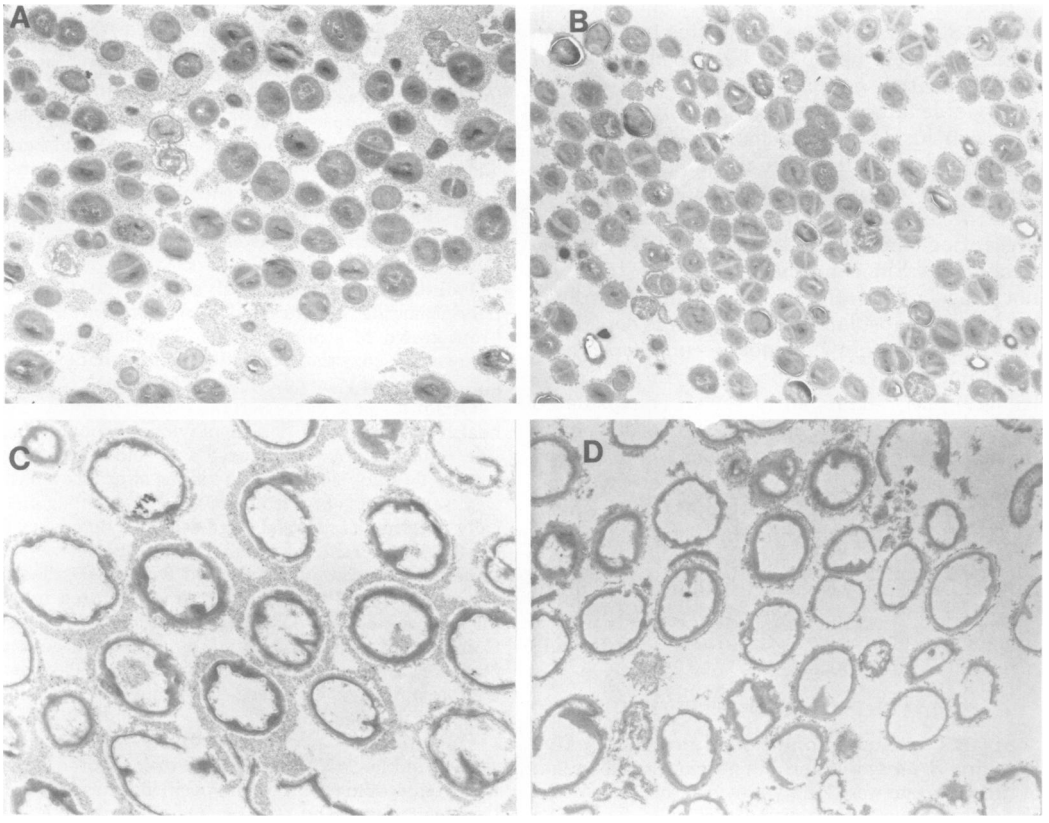

FIG. 1. *Electron micrographs of S. aureus M (A), S. aureus M variant (B), CW+C (C), and PG (D) after incubation in S. aureus M immune rabbit serum. (A) and (B) × 3,900; (C) and (D) × 7,800.*

from a rabbit immunized with heat-killed *S. aureus* M (13, 19). Capsular material can be clearly seen on the surface of the intact M strain and CW+C preparations, but not on the PG preparations or on the unencapsulated M variant strain. In a slide agglutination test, the CW+C preparations were agglutinated by *S. aureus* M immune serum, whereas PG from strain M and both purified and crude cell walls from *S. aureus* H (18) were not. Amino acid analysis of a 6 M HCl, 18-h 105°C hydrolysate revealed the following components to be present in a CW+C preparation (in nanomoles per milligram, dry weight): muramic acid, 442; glucosamine, 666; alanine, 1,431; glutamic acid, 567; lysine, 543; glycine, 2,673. There were 580 nmol of phosphate per mg of CW+C (dry weight). When a formula weight of 1,138 for the PG repeating unit (18) and the lysine figure are used, the CW+C preparations are composed of roughly 62% PG, with teichoic acid, capsular material, and counterions to negatively charged groups making up the rest of the weight of the preparation. Analyses showed that most of the weight of the PG preparation was accountable for as PG components (in nanomoles per milligram, dry weight): muramic acid, 274; glucosamine, 281; alanine 2,288; glutamic acid, 919; lysine, 856; glycine, 3,516. Hot trichloroacetic acid treatment removed about 40% of the weight of the CW+C preparation and 90% of the phosphate (a teichoic acid marker).

The capsular polysaccharide has been reported to be composed of *N*-acetyl-D-aminogalacturonic acid, *N*-acetyl-D-fucosamine, and taurine (11). It was hoped that taurine could be used as a marker for the presence or the absence of capsular material in our preparations because of the stability of taurine to acid hydrolysis and its distinctive elution position from the amino acid analyzer. Surprisingly, taurine could not be detected in analyses of CW+C, but was detected upon analysis of partially purified polysaccharide isolated from M strain grown in brain heart infusion broth, the growth medium used in the original publication of Liau et al. (11). The presence of taurine in the capsule appears to be dietary dependent since amino acid analysis

after acid hydrolysis revealed no taurine in phytone peptone or yeast extract, which are components of the medium used in this study. However, 8 nmol of taurine per mg (dry weight) was detected upon analysis of brain heart infusion powder. Nevertheless, two components, eluted on the analyzer closely after glucosamine, were detected in analyses of both partially purified polysaccharide and CW+C, but not PG. These components were tentatively identified as fucosamine and aminogalacturonyl fucosamine (11). Thus, the chemical, immunological, and electron microscopic evidence supports the identification of the preparations as CW+C and PG.

Opsonization of *S. aureus* M, M variant, CW+C and PG in normal human serum. Figure 2 shows the PMN uptake of [2-^{3}H]glycine-labeled strain M cells, CW+C, and PG after incubation in normal and heat-inactivated sera. These sera were poor opsonic sources for the intact bacteria as they mediated little phagocytosis. Under these conditions there was 54 and 6% phagocytosis of the unencapsulated M variant strain (data not shown) after incubation in normal and heat-inactivated sera, respectively. This demonstrates that the encapsulated organism is not efficiently opsonized by factors in normal human serum and that a variant of this organism which lacks a capsule is well opsonized under the same conditions.

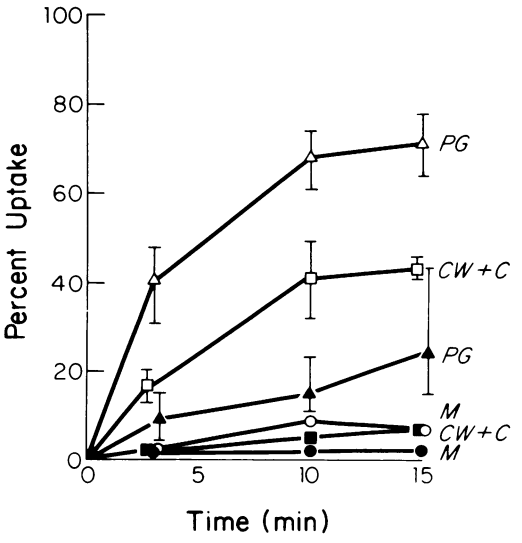

FIG. 2. *Phagocytosis of S. aureus M, CW+C, and PG by PMNs after incubation in human serum. Particles were incubated for 5 min in 10% normal human serum (open symbols) or heat-inactivated serum (solid symbols) before being presented to PMNs. Phagocytosis was quantitated by measuring leukocyte-associated radioactivity. Symbols represent the means of three experiments done on 3 separate days; bars represent the ranges.*

However, PG isolated from strain M was well opsonized by normal serum as revealed by efficient phagocytosis of this preparation (Fig. 2). CW+C were phagocytized, but at a slower rate and to a lesser extent than was PG (Fig. 2). Heat-inactivated serum was a poor opsonic source, although there was significantly greater uptake of PG after incubation in this opsonic source than of intact bacteria or CW+C. Opsonization and phagocytosis of PG isolated from *S. aureus* M were similar to those of the intact, unencapsulated M variant strain and of PG isolated from *S. aureus* H (an unencapsulated strain) (18). These observations demonstrate that the encapsulated organism contains a cryptic, opsonizable subcellular structure, PG. It appears that the capsule interferes with opsonization of PG and thereby accounts for the poor phagocytosis of the intact encapsulated bacteria. Further evidence to support this conclusion is the finding that CW+C were not as efficiently opsonized as PG. The removal of capsular material from CW+C by hot trichloroacetic acid treatment yielded a particle (i.e., PG) that was more efficiently opsonized. This treatment removes teichoic acid, in addition to capsular material, from cell walls. However, removal of teichoic acid from cell walls was previously shown not to alter the rate or the extent of phagocytosis of these particles (18). The opsonization and phagocytosis of CW+C that did occur may be explained by the presence of particles not retaining capsular material and exposure of non-capsule-covered surfaces in this preparation.

Opsonization of *S. aureus* M, CW+C, and PG in the absence of an intact classical complement pathway. To study opsonization via the alternative complement pathway, C2-deficient serum was used, and the opsonic capacity of this serum was compared with that of normal serum. When intact bacteria, CW+C, and PG were opsonized for only 5 min in C2-deficient serum, there was poor phagocytosis of all three particle types (Table 1). Incubation of PG, CW+C, and *S. aureus* M for 5 min in normal human serum led to phagocytosis little different from particles incubated in this serum for 60 min (Fig. 2). However, when incubated for 60 min in C2-deficient serum, there was 55, 29, and 7% uptake of PG, CW+C, and *S. aureus* M, respectively, after 15 min of incubation with PMNs. These results indicate that PG isolated from *S. aureus* M can be opsonized via the alternative pathway, although at a significantly slower rate than when the classical pathway is present (normal serum), and that capsular material inter-

TABLE 1. *Phagocytosis of S. aureus M, CW+C, and PG by PMNs after incubation in C2-deficient serum*[a]

Particle	Incubation time (min) in serum	% Phagocytosis (15 min)
S. aureus M	5	3
	60	7
CW+C	5	11
	60	29
PG	5	17
	60	55

[a] Particles were incubated for indicated times in 10% C2-deficient serum before being presented to PMNs. Phagocytosis was quantitated by measuring leukocyte-associated radioactivity. Results represent the means of three experiments done on 3 separate days.

feres with opsonization via both the classical and the alternative complement pathways.

Opsonization of *S. aureus* M, CW+C, and PG in *S. aureus* M immune rabbit serum. The only effective opsonic source for *S. aureus* M has been serum obtained from animals immunized with this bacterium (13, 19). In this study, *S. aureus* M, CW+C, and PG were incubated with heat-inactivated preimmune and immune rabbit sera. There was less than 10% uptake of all three particle types by PMNs after 15 min after incubation of the particles in heat-inactivated preimmune serum (Fig. 3). When heat-inactivated immune serum was used as an opsonic source, there was 47 and 66% uptake of *S. aureus* M and CW+C, respectively, compared with only 10% uptake of PG. These findings demonstrate that a heat-stable, capsule-directed serum factor(s), presumably antibody, is necessary to promote effective opsonization of the encapsulated organism and that this same factor(s) promotes opsonization of CW+C but not of PG.

DISCUSSION

The major purpose of this study was to examine the basis of the antiphagocytic effect of the *S. aureus* capsule. However, before the results of this investigation are discussed, it is important to clarify the meaning of the term antiphagocytic.

In early studies correlations were noted between the presence and the absence of certain cell surface polymers in a particular bacterial species and resistance to phagocytosis and virulence. This led to the description of these polymers as antiphagocytic, classical examples of which are the *Streptococcus pneumoniae* capsule (1) and the M protein of the group A streptococcus (9). The ability of antiphagocytic antigens to remove type-specific opsonic activity from antisera has been used to confirm the antiphagocytic effect of a particular antigen (12), and the term antiphagocytic is applied generally to bacterial products which remove opsonic activity from serum. The term antiphagocytic has also been applied to bacterial products which depress phagocytosis when added to a test system (5). In phagocytic systems where serum is present, this effect may be due to competition for serum opsonins with the test particle since capsular material from a wide variety of bacterial species is relatively noncytotoxic (21).

Thus, as originally conceived, an antiphagocytic polymer is one that confers resistance to phagocytosis upon a microorganism. At this point it is necesary to distinguish between phagocytosis occurring in the absence of opsonins, of which the "surface phagocytosis" described by Wood and Smith was an early example (28), and the generally more rapid phagocytosis mediated primarily through opsonins (24), with which we are concerned here. From a physicochemical standpoint it is believed that both a hydrophilic and a negatively charged particle surface retard phagocytosis (14, 25). Thus, in this sense a particular cell surface polymer may, by its chemical nature, be regarded as inherently antiphagocytic

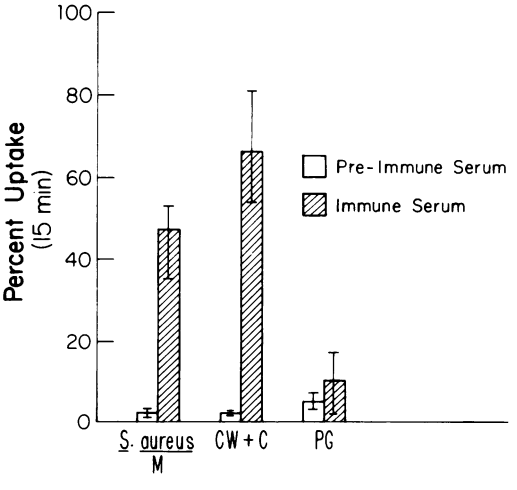

FIG. 3. *Phagocytosis of S. aureus M, CW+C, and PG by PMNs after incubation in rabbit serum. Particles were incubated in 10% heat-inactivated preimmune or S. aureus M immune rabbit serum for 15 min before being presented to PMNs. Phagocytosis was quantitated by measuring leukocyte-associated radioactivity. Bars represent the means of three experiments done on 3 separate days; brackets represent the ranges.*

and the function of opsonins viewed as functioning in such a manner that this antiphagocytic property is negated. However, current interpretations of opsonin-mediated phagocytosis are that it is not so much that opsonins cover regions of a particle which inherently resist recognition and ingestion but that bound opsonins themselves constitute favorable configurations which are recognized by phagocytic cells (24).

Previous studies led us to the conclusion that recognition of unencapsulated *S. aureus* organisms is mediated via opsonization of surface-exposed, cell wall PG (18). Encapsulated bacteria, however, have an additional cell surface polymer external to the cell wall. Results of experiments reported in this study support the hypothesis that encapsulated *S. aureus* can evade the process of opsonization and that this phenomenon is due to masking of the cell wall PG by the capsule. Hence, the antiphagocytic property of the capsule may reside in its ability to interfere with the process of opsonization.

In an earlier study (19) we found that encapsulated strains were inefficiently opsonized by normal human serum in contrast to unencapsulated variants of these strains. Although intact *S. aureus* M cells are essentially not opsonized by normal serum, in this study it was found that PG isolated from this organism was readily opsonized and phagocytized by human PMNs. This observation suggests that PG in encapsulated bacteria is cryptic or hidden from effective interaction with opsonins present in normal serum. Isolated CW+C preparations were phagocytized at about half the rate and to half the extent of PG after opsonization of these particles in normal serum. Removal of capsular material by hot trichloroacetic acid treatment yielded a particle (PG) that was more efficiently opsonized. The inner surface of the cell wall is exposed in CW+C preparations, and this is thought to be a possible explanation as to why these particles were more efficiently opsonized than were whole organisms.

Efficient opsonization of the encapsulated *S. aureus* M apparently requires type-specific antibodies to be present in serum. Heat-inactivated immune rabbit serum was found to have an increased opsonic capacity for the intact organism and CW+C but not for a particle that lacks capsular antigen (PG).

Currently, it is thought that bacterial phagocytosis by PMNs is mediated via plasma membrane receptors with specificities for the Fc portion of antibody molecules and for the C3b fragment of complement bound to the surface of the bacterium (20, 26). The mechanism by which the capsule masks PG from salutary interaction with these opsonins in normal serum is unclear at this time. There are at least two possible explanations for this effect: (i) the capsule acts as a physicochemical barrier, preventing access of opsonic factors to PG; (ii) the capsule, in some way, interferes with the opsonic effect of serum factors, even though they become bound to the bacterial cell surface. In either case, the capsule interferes with the normal recognition of the bacterium via leukocyte receptors.

There is limited information available as to a possible barrier effect of the staphylococcal capsule. Bacteriophages apparently are excluded from interaction with their receptor (cell wall PG and teichoic acid) by the capsule since staphylococcal phages have been found to inefficiently adsorb to encapsulated staphylococcal strains in contrast to related unencapsulated variants (27). However, the staphylococcal capsule appears to pose no barrier to homologous antibody (Fig. 1) nor to the small-molecular-weight protein lysostaphin (13).

From observations in a previous study (17), it does seem that complement is not strictly excluded from an interaction with the staphylococcal cell surface, for it was found that encapsulated and unencapsulated *S. aureus* strains activated complement to a similar extent and that both stained positively with anti-C3 fluorescent antibody. This finding indicates that C3 is present on the encapsulated staphylococcal cell surface, but does not tell us where the C3 is located or how it is displayed. Perhaps the capsule interferes with exposure of opsonic factors in the proper configuration at the true external surface of the bacterium.

PG is a major cell wall component of most gram-positive bacterial species, and the cell surface of such organisms may be most accurately viewed as a mosaic of different domains, where PG is sufficiently exposed to interact with external factors (8, 18). Several features of the chemical composition, structure, and biological properties of PG are shared by this polymer in a wide variety of bacteria. Heymer and Reitschel (2) have pointed out that the occurrence of PG in nature is as widespread as that of bacteria and that the frequency of detection of PG antibodies in normal sera of animals and humans depends primarily on the sensitivity of the methods employed. Others have speculated that host reaction to PG may form the basis of natural immunity to gram-positive bacteria (4, 7). Recognition of many gram-positive bacterial species may, therefore, be a manifestation of host response to PG.

It is possible that the presence of capsular material in gram-positive species other than *S. aureus*, e.g., *S. pneumoniae*, masks PG from opsonins present in normal human serum and

thereby interferes with natural immunity. Likewise, cell surface polymers such as the M protein of the group A streptococcus, which has long been recognized as antiphagocytic (9), may interfere with opsonization of PG in a similar manner. It also seems likely that the masking effect of a capsule pertains to the antiphagocytic characteristic of such material in gram-negative bacterial species. The capsular antigens of this group of organisms may mask some common outer membrane component yet to be defined.

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