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Influence of Encapsulation on Staphylococcal Opsonization and Phagocytosis by Human Polymorphonuclear Leukocytes

PHILLIP K. PETERSON,[1]* BRIAN J. WILKINSON,[1,2] YOUNGKI KIM,[3] DAVID SCHMELING,[3] AND PAUL G. QUIE[3]

Departments of Medicine,[1] Pediatrics,[3] and Microbiology,[2] University of Minnesota School of Medicine, Minneapolis, Minnesota 55455

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In previous studies, encapsulated *Staphylococcus aureus* strains have been shown to resist phagocytosis. In this investigation, the nature of the interference with phagocytosis by human polymorphonuclear leukocytes was examined by studying the opsonization of two pairs of unencapsulated (Smith compact and M variant) and encapsulated (Smith diffuse and M) *S. aureus* strains. The uptake of [^{3}H]glycine-labeled bacteria by normal leukocytes was quantitatively measured after incubation of bacteria in pooled serum, C2-deficient serum, immunoglobulin-deficient serum, and serum from a rabbit immunized with *S. aureus* M. The presence of a capsule was found to interfere with opsonization by both the classical and alternative pathways of complement as well as by heat-stable opsonic factors in nonimmune human serum. This interference was significantly greater in the case of the *S. aureus* M strain than in the case of the Smith diffuse strain. The only effective opsonic source for *S. aureus* M was immune rabbit serum. It is proposed that encapsulation of *S. aureus* strains interferes with phagocytosis by preventing effective bacterial opsonization.

The cell wall of most *Staphylococcus aureus* strains is composed of three major constituents—peptidoglycan, teichoic acid, and protein A. The last component has been demonstrated to interfere with staphylococcal opsonization in vitro (3, 9), and, although in vivo support is lacking, protein A has been proposed as a staphylococcal virulence factor. A polysaccharide capsule is an additional component of the cell surface of some *S. aureus* strains. Although encapsulated staphylococci are generally considered rare, there is considerable evidence to suggest that this may not be the case in vivo (6–8, 11, 18, 20, 22, 24, 25). That the capsule can function as a significant staphylococcal virulence factor has been demonstrated both in vitro and in vivo (7, 8, 23, 26).

Although the exact mechanism by which the capsule functions as a virulence factor has not been established, it has been shown that encapsulated staphylococci resist phagocytosis, a phenomenon that may be related to the special opsonic requirements of these strains (8, 13). In the present investigation, the serum factors required for phagocytosis of two pairs of encapsulated and unencapsulated *S. aureus* strains were studied. Radioactively labeled bacteria and human polymorphonuclear leukocytes (PMNs) were used in a quantitative assay system.

MATERIALS AND METHODS

Bacterial strains and radioactive labeling. *S. aureus* Smith compact (unencapsulated) and diffuse (encapsulated) strains and *S. aureus* M variant (unencapsulated) and M (encapsulated) strains were kindly provided by M. A. Melly, Vanderbilt School of Medicine, Nashville, Tenn. Descriptions and classifications of three of these strains have been presented elsewhere (8, 14, 25). The *S. aureus* M variant strain produces nonmucoid colonies and does not possess a visible capsule; however, it has some of the properties of the encapsulated M strain (clumping factor negative, diffuse colonies in serum-soft agar; M. A. Melly, personal communication). *S. aureus* Cowan I and a clinical isolate of *Pseudomonas aeruginosa* were also used. All strains were maintained on nutrient agar plates at 4°C. Radioactive labeling of the *S. aureus* strains was achieved by inoculating several colonies into 20 ml of peptone-yeast extract broth (4) containing 0.04 mCi of [2-^{3}H]glycine (specific activity, 5 to 15 Ci/mmol, New England Nuclear Corp., Boston, Mass); the pseudomonas strain was labeled by inoculating several colonies into 20 ml of Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) containing 0.04 mCi of [2-^{3}H]adenine (specific activity, 5 to 15 Ci/mmol, New England Nuclear Corp.). After 18 h of incubation at 37°C, the bacteria were washed three times in phosphate-buffered saline (pH 7.4) and resuspended in phosphate-buffered saline to a final concentration of 10^9 colony-forming units/ml (determined by a spectrophotometric method and confirmed by pour plate colony counts).

Opsonic sources. Serum was collected from four healthy donors and pooled. To study opsonization in the absence of an intact classical pathway of complement, serum was obtained from a donor with a genetically determined complete and selective deficiency of C2 (5). Opsonization in the relative absence of antibodies was investigated with sera from two donors: D.S., a 62-year-old male with common variable immunodeficiency (immunoglobulin G, 73 mg/100 ml; immunoglobulin M, 7 mg/100 ml; and immunoglobulin A, <3 mg/100 ml by laser nephelometry), and D.R., a 24-year-old male with X-linked infantile agammaglobulinemia (immunoglobulin G, 98 mg/100 ml; immunoglobulin M, <10 mg/100 ml; and immunoglobulin A, <3 mg/100 ml). To study opsonization in immune serum, an adult New Zealand white rabbit was injected through an ear vein with 0.5 ml of staphylococcal M vaccine twice a week for 3 weeks; serum was collected before immunization and 1 and 4 weeks after immunization was completed. The rabbit was boosted with 0.5 ml of vaccine 4 days before the final serum was taken. The vaccine was broth-grown M strain (1.25×10^{10} colony-forming units/ml), washed four times in physiological saline and heat killed by autoclaving (20 min at 121°C). All serum sources were stored in 0.5-ml portions at −70°C and thawed just before use. Heat-inactivated serum was prepared by heating thawed portions at 56°C for 30 min. Serum was diluted to specified concentrations with Hanks balanced salt solution containing 0.1% gelatin.

Opsonization procedure. A 0.10-ml portion of each bacterial suspension was incubated at 37°C in polypropylene vials (Bio-vials; Beckman, Chicago, Ill.) containing 1.0 ml of the indicated opsonic source for specified time intervals in an incubator shaker (New Brunswick Scientific, New Brunswick, N.J.) at 250 rpm; 2.0 ml of ice-cold phosphate-buffered saline was added, and the mixture was centrifuged at $2,000 \times g$ for 15 min (4°C). The supernatant was then discarded, and the bacterial pellet was suspended vigorously in 1.0 ml of Hanks balanced salt solution.

PMNs. Venous blood was collected from healthy donors in heparinized syringes (10 U of heparin per ml of blood). Suspensions of pure PMNs were prepared with a method modified from Böyum (1) that employed a Ficoll-Isopaque gradient to separate mononuclear and polymorphonuclear cells and hypotonic NH_4Cl to lyse erythrocytes (10). PMNs were washed four times to eliminate accompanying complement components (12). Final leukocyte suspensions were adjusted to a concentration of 10^7 PMNs per ml of Hanks balanced salt solution.

Phagocytosis mixtures and assays. Bacterial uptake by PMNs was determined with a modification of a previously described method (17). Briefly, 100-μl samples from the suspension of opsonized bacteria followed by 100-μl samples of the leukocyte suspension were added to polypropylene vials, yielding a colony-forming units/PMN ratio of approximately 10:1. The vials were then incubated for specified time intervals at 37°C in the incubator shaker at 250 rpm. Immediately after removal from the incubator, 3.0 ml of ice-cold phosphate-buffered saline was added to each vial. Leukocyte-associated radioactivity was determined after the PMNs were washed three times in cold phosphate-buffered saline by means of differential centrifugation at $160 \times g$ (4°C). The final leukocyte pellets were suspended in 3.0 ml of scintillation liquid (Aquasol-2; New England Nuclear Corp.) and counted in a liquid scintillation counter (Beckman LS-250). To determine total radioactivity (representing both leukocyte-associated and non-leukocyte-associated bacteria), vials were prepared as outlined above and removed from the incubator at indicated times. After 3.0 ml of phosphate-buffered saline was added, the vials were centrifuged at $2,000 \times g$ for 15 min. Supernatants were discarded, and the pellets were suspended in 3.0 ml of scintillation liquid and counted in the scintillation counter. Samples were taken in duplicate, and an average of the duplicate values was used in the calculations. All experiments were repeated on at least three separate days with PMNs from different donors. The uptake of bacteria by PMNs at a given sampling time was calculated with the formula:

$$\% \text{ uptake} = \frac{\text{counts per minute in leukocyte pellet}}{\text{total counts per minute}} \times 100$$

Control experiments, as outlined by Stossel (15), demonstrated that (i) as the bacteria/PMN ratio was increased, the rate of uptake was saturable; (ii) there was no uptake at zero time (samples processed immediately after constituting the phagocytosis mixture); and (iii) there was essentially no uptake under ice-bath conditions. All of these findings indicated that bacterial ingestion was being measured. A morphological evaluation of ingestion was also performed by taking 50-μl samples from vials processed in the above manner, depositing the PMNs on glass slides with a cytocentrifuge, and, after staining with Wright strain, examining the PMNs under a light microscope. Although the results obtained with the microscopic method were not as quantitatively accurate, they correlated well with the findings obtained with the method described above.

RESULTS

Kinetics of phagocytosis of *S. aureus* Smith and M strains. After incubation of the *S. aureus* Smith compact (unencapsulated), Smith diffuse (encapsulated), M variant (unencapsulated), and M (encapsulated) strains in 10% pooled serum and 10% heat-inactivated pooled serum for 5 min, phagocytosis mixtures were constituted, and uptake by PMNs was measured at 3, 10, and 15 min (Fig. 1). When pooled serum was used as an opsonic source, both encapsulated strains were poorly phagocytized when compared with their unencapsulated counterpart strains. Whereas there was approximately 85% uptake of the two unencapsulated strains at 15 min, there was 51% uptake of the Smith diffuse strain and only 26% uptake of the M strain. When heat-inactivated serum was used as an opsonic source, there was poor uptake of the Smith diffuse strain and both M strains

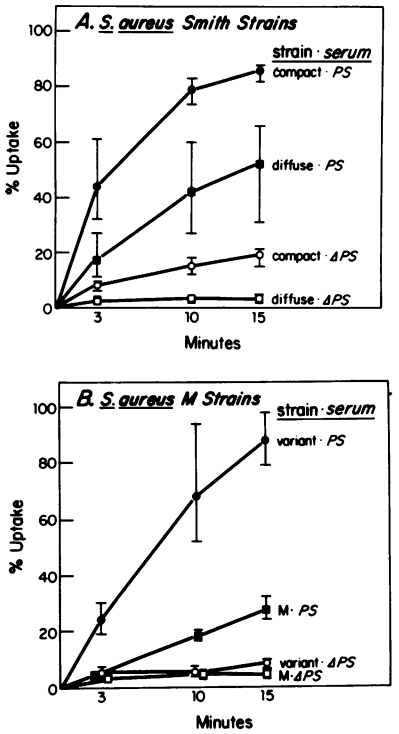

FIG. 1. *Phagocytosis of S. aureus Smith compact and Smith diffuse strains (A) and S. aureus M variant and M strains (B) after opsonization for 5 min in 10% concentrations of pooled serum (PS) and heat-inactivated pooled serum (ΔPS). Uptake by PMNs was determined at 3, 10, and 15 min. Results represent the means of three experiments; brackets represent ranges.*

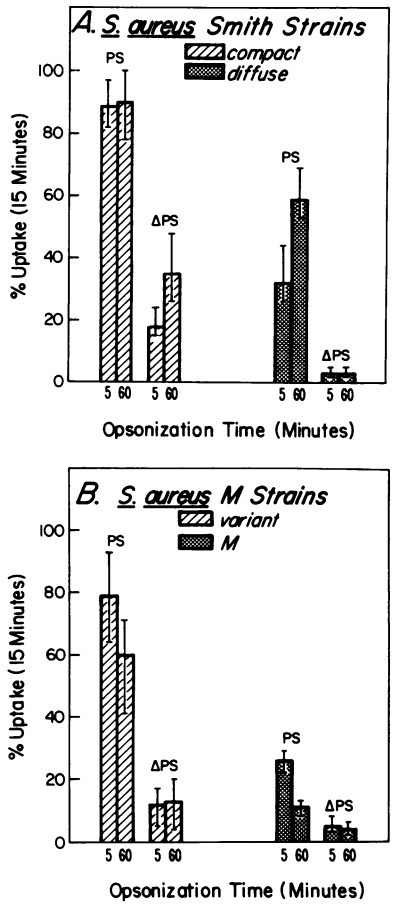

FIG. 2. *Phagocytosis of S. aureus Smith compact and Smith diffuse strains (A) and S. aureus M variant and M strains (B) after opsonization for 5 and 60 min in 10% concentrations of pooled serum (PS) and heat-inactivated pooled serum (ΔPS). Uptake by PMNs was determined at 15 min. Results represent the means of three experiments; brackets represent ranges.*

(<10% uptake at 15 min). Heated serum was also a poor opsonic source for the Smith compact strain; however, there was greater uptake than that found with the other strains (19% uptake at 15 min).

Influence of opsonization time on phagocytosis. To study whether a more prolonged period of opsonization might enhance subsequent phagocytosis of the encapsulated strains, bacteria were incubated for 60- and 5-min periods in 10% concentrations of pooled serum and heat-inactivated serum before phagocytosis mixtures were constituted and uptake was measured at 15 min (Fig. 2). The more prolonged, 60-min period of opsonization did result in increased phagocytosis of the encapsulated Smith diffuse strain when pooled serum was used; however, uptake was still significantly less than that of the Smith compact strain (Fig. 2A). When heated serum was used, increasing the opsonization period from 5 to 60 min resulted in increased phagocytosis of the Smith compact strain (18 and 35% uptake, respectively); however, there was essentially no uptake of the Smith diffuse strain when opsonized for either time period. The more prolonged opsonization period did not enhance phagocytosis of the two *S. aureus* M strains when either pooled serum or heated serum was studied (Fig. 2B). Indeed, when pooled serum was used as an opsonic source, there was greater uptake of bacteria opsonized for 5 min than for 60 min (79 versus 60% uptake of the M variant strain, 26 versus 11% uptake of the M strain).

Influence of serum concentration on phagocytosis. To evaluate the effect of increasing the serum concentration on phagocytosis, bacteria were incubated for 5 min in 100, 25, 10,

1.0, and 0.1% concentrations of pooled serum and heat-inactivated serum before phagocytosis mixtures were constituted and uptake was measured at 15 min (Fig. 3). Increasing the concentration of pooled serum had a significant enhancing effect on phagocytosis of the *S. aureus* Smith diffuse strain. When 25 and 100% concentrations of pooled serum were used, there was similar uptake of the two Smith strains (Fig. 3A). However, there was poor uptake of the encapsulated *S. aureus* M strain with all serum concentrations (12% uptake of bacteria opsonized with 25 and 100% concentrations of pooled serum; Fig. 3B). When heated serum was used as an opsonic source, there was again a significant enhancement of phagocytosis of the encapsulated Smith diffuse strain as the concentration of serum was increased (53% uptake of Smith diffuse versus 62% uptake of the Smith compact strain when 100% heated serum was used). Although there was increased phagocytosis of the M variant strain as the concentration of heated serum was increased, there was no enhancement of uptake of the encapsulated M strain (21% uptake of M variant versus 4% uptake of M with undiluted heated serum; data not shown). These findings and the preceding results demonstrate that, whereas both encapsulated *S. aureus* strains were (in general) poorly phagocytized after opsonization in normal human serum and heat-inactivated serum, opsonization of the Smith diffuse strain could be significantly enhanced by increasing the duration of opsonization and the serum concentration; however, opsonization of the encapsulated M strain could not be increased by these methods.

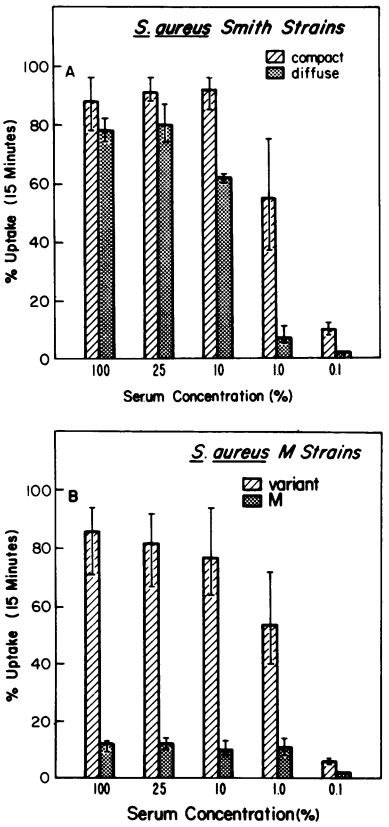

FIG. 3. *Phagocytosis of S. aureus Smith compact and Smith diffuse strains (A) and S. aureus M variant and M strains (B) after opsonization for 5 min in 100, 25, 10, 1.0, and 0.1% concentrations of pooled serum. Uptake by PMNs was determined at 15 min. Results represent the means of three experiments; brackets represent ranges.*

Opsonization in the absence of an intact classical complement pathway. To study the role of the classical complement pathway in opsonization, bacteria were incubated for 5 and 60 min in 10% concentrations of pooled serum, C2-deficient serum, and heat-inactivated serum before constituting the phagocytosis mixtures and measuring the uptake at 15 min (Fig. 4 and 5). C2-deficient serum was a poor opsonic source for both encapsulated strains. After 60 min of opsonization, there was only 12 and 6% uptake of the Smith diffuse and M strains, respectively, compared with 65 and 68% uptake of the Smith compact and M variant strains, respectively. Opsonization of the unencapsulated strains proceeded at a significantly slower rate in the absence of the classical pathway (C2-deficient serum) than in its presence (pooled serum).

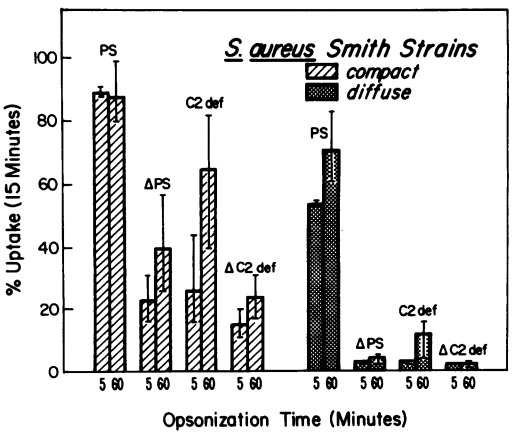

FIG. 4. *Phagocytosis of S. aureus Smith compact and Smith diffuse strains after opsonization for 5 and 60 min in 10% concentrations of pooled serum (PS), heat-inactivated pooled serum (ΔPS), C2-deficient serum (C2 def), and heat-inactivated C2-deficient serum (ΔC2 def). Uptake by PMNs was determined at 15 min. Results represent the means of three experiments; brackets represent ranges.*

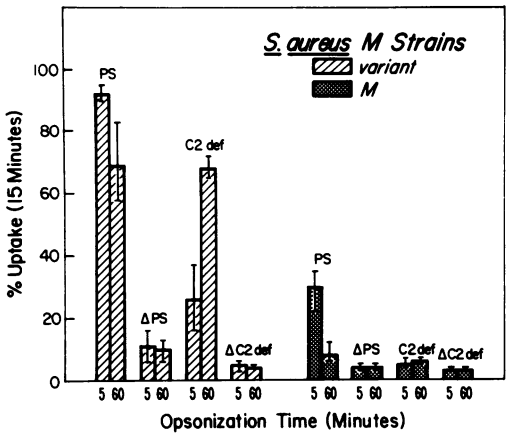

FIG. 5. *Phagocytosis of S. aureus M variant and M strains after opsonization for 5 and 60 min in 10% concentrations of pooled serum (PS), heat-inactivated pooled serum (ΔPS), C2-deficient serum (C2 def), and heat-inactivated C2-deficient serum (ΔC2 def). Uptake by PMNs was determined at 15 min. Results represent the means of three experiments; brackets represent ranges.*

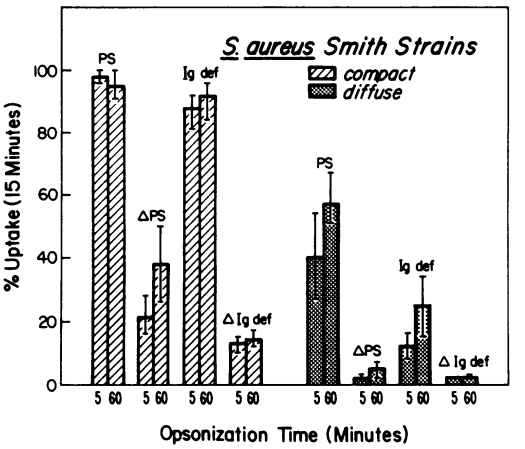

FIG. 6. *Phagocytosis of S. aureus Smith compact and Smith diffuse strains after opsonization for 5 and 60 min in 10% concentrations of pooled serum (PS), heat-inactivated pooled serum (ΔPS), immunoglobulin-deficient serum (Ig def), and heat-inactivated, immunoglobulin-deficient serum (ΔIg def). Uptake by PMNs was determined at 15 min. Results represent the means of three experiments; brackets represent ranges.*

Whereas there was approximately 90% uptake of the Smith compact and M variant strains after 5 min of opsonization in pooled serum, only about 25% of these bacteria were phagocytized after 5 min of opsonization in C2-deficient serum. Opsonization for 60 min significantly increased the uptake of both strains when C2-deficient serum was used as an opsonic source, but not when pooled serum was used. These findings suggest that encapsulation impedes opsonization by both the classical and the alternative pathways of complement.

Opsonization in the relative absence of immunoglobulin. The role of immunoglobulins in opsonization was studied by incubating bacteria for 5 and 60 min in 10% concentrations of pooled serum, immunoglobulin-deficient serum (donor D.S.), and heat-inactivated serum before constituting the phagocytosis mixtures and measuring the uptake at 15 min (Fig. 6 and 7). Whereas immunoglobulin-deficient serum was a good opsonic source for both unencapsulated strains, there was poor uptake of the two encapsulated strains after incubation in this serum source. Similar findings were obtained when immunoglobulin-deficient serum from donor D.R. was studied (data not presented). These results demonstrate that unencapsulated bacteria can be opsonized by heat-labile serum factors in the relative absence of antibodies and that encapsulation interferes with opsonization in this serum source.

Opsonization in heat-inactivated immune rabbit serum. The opsonic capacity of

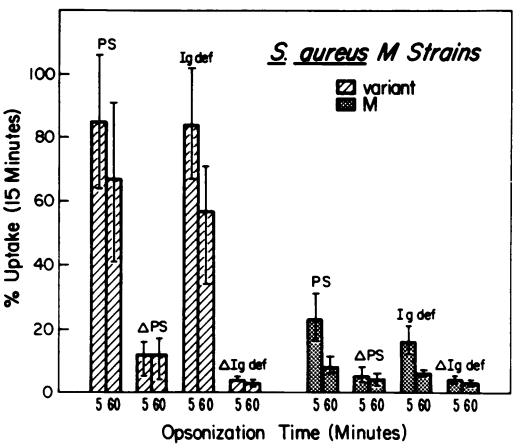

FIG. 7. *Phagocytosis of S. aureus M variant and M strains after opsonization for 5 and 60 min in 10% concentrations of pooled serum (PS), heat-inactivated pooled serum (ΔPS), immunoglobulin-deficient serum (Ig def), and heat-inactivated, immunoglobulin-deficient serum (ΔIg def). Uptake by PMNs was determined at 15 min. Results represent the means of three experiments; brackets represent ranges.*

serum obtained from a rabbit immunized with *S. aureus* M for the encapsulated M, M variant, and Cowan I strains and a pseudomonas strain was tested by incubating these bacterial strains for 5 min in 10% concentrations of heat-inactivated preimmune and immune sera (collected 1 and 4 weeks after immunization was completed)

before constituting the phagocytosis mixtures and determining the uptake at 15 min (Fig. 8). Heated preimmune serum was a poor opsonic source for all four bacterial strains. Serum obtained 1 week after immunization had a marked increase in heat-stable opsonic activity for both M strains but not for *S. aureus* Cowan I or the pseudomonas strain. Whereas the serum obtained 4 weeks after immunization continued to display significant heat-stable opsonic activity for the M variant strain, it was a poor opsonic source for the M strain. These findings suggest that immunization with the encapsulated M strain resulted in the production of heat-stable serum factors (most probably antibodies) that were opsonic for the two M strains and that these heat-stable factors may not have been directed against a common antigen.

DISCUSSION

In 1903, Wright and Douglas (21) established the critical role played by serum factors in promoting staphylococcal phagocytosis and provided us with the definition of "opsonins"—"... elements in the blood which ... modify the bacteria in a manner which renders them a ready prey to the phagocytes." In the present investigation, evidence has been given to support the concept that, by this definition, encapsulation interferes with effective opsonization. Encapsulated staphylococci (*S. aureus* Smith diffuse and M strains) are not as "appealing" as unencapsulated bacteria (*S. aureus* Smith compact and M variant strains) to human PMNs after incubation of these bacterial strains in a variety of opsonic sources.

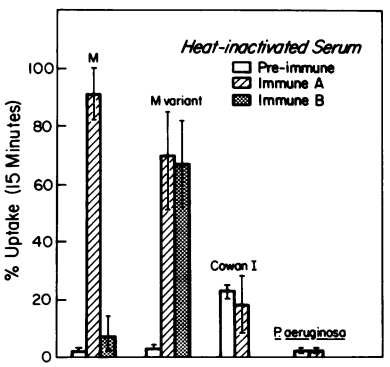

FIG. 8. *Phagocytosis of S. aureus strains M, M variant, and Cowan I and P. aeruginosa after opsonization for 5 min in 10% concentrations of heat-inactivated preimmune rabbit serum and rabbit serum obtained 1 week (A) and 4 weeks (B) after the completion of immunization. Uptake by PMNs was determined at 15 min. Results represent the means of the three experiments; brackets represent ranges.*

The opsonic requirements for phagocytosis of the two unencapsulated strains used in this study were found to be similar to those of other *S. aureus* strains (16). Optimal opsonization of these strains required the presence of an intact complement system. In the absence of the classical complement pathway (C2-deficient serum), opsonization occurred via the alternative complement pathway; however, opsonization was significantly slower and less effective than when the classical pathway was present (pooled normal serum). Sera from two donors with immunoglobulin deficiency states were as effective as opsonic sources as normal serum, suggesting that antibodies may not be necessary for opsonization of these strains. Heat-inactivated serum was moderately opsonic for the Smith compact strain and, when used in an undiluted concentration, was somewhat opsonic for the M variant strain.

When opsonization of the two encapsulated strains was compared with that of their unencapsulated counterpart strains, significant differences were observed. In addition, marked differences were found when opsonization of the two encapsulated strains was compared. The presence of a capsule interfered with opsonization via the classical and alternative pathways of complement (pooled serum, immunoglobulin-deficient serum and C2-deficient serum) and via heat-stable serum factors present in nonimmune human serum. Diminished phagocytosis of the Smith diffuse strain was most pronounced when a serum concentration of 10% was used and when the opsonization period was limited to 5 min. By increasing the serum concentration and the duration of opsonization to 60 min, phagocytosis of the encapsulated Smith diffuse strain approached that of the unencapsulated Smith compact strain. Phagocytosis of the encapsulated M strain remained significantly depressed regardless of the serum concentration used and the duration of the opsonization period.

These findings corroborate the observations of Melly et al. (8), who showed, in a mouse peritoneal model, that the 50% lethal dose of *S. aureus* M was 100-fold less than that of *S. aureus* Smith diffuse and (with a microscopic method) that the M strain was less readily phagocytized by human PMNs than the Smith diffuse strain. These authors suggested that the more marked virulence of the M strain correlated with the larger size of its capsule. In studies of other encapsulated *S. aureus* strains, similar findings and conclusions have been reported by Yoshida and Takeuchi (26).

The only serum that served as an effective opsonic source for the encapsulated *S. aureus* M strain was obtained from a rabbit 1 week after

immunization with heat-killed M bacteria. Heat-inactivated immune rabbit serum was also found to be opsonic for the unencapsulated M variant strain but not for another *S. aureus* strain or for a *P. aeruginosa* strain. When serum was collected 1 month after immunization, the heat-stable opsonic activity had become markedly diminished for the M strain but not for the M variant strain. This finding suggested the possibility that anticapsular antibodies were responsible for the enhanced opsonization of the M strain, and that antibodies directed against another cell surface component were opsonic for the M variant strain. Additional studies will be necessary to define the nature of these antibodies. These results are in general agreement with those of other investigators who have shown that capsular antibodies are protective against infection by encapsulated strains (2, 8, 19).

Recent work in this laboratory has demonstrated that the peptidoglycan component of the *S. aureus* cell wall is the key constituent involved in promoting opsonization by serum factors in nonimmune human serum (P. K. Peterson, B. J. Wilkinson, Y. Kim, J. Verhoef, D. Schmeling, S. D. Douglas, and P. G. Quie, J. Clin. Invest., in press). It is proposed that a capsule interferes with staphylococcal opsonization by covering cell wall peptidoglycan and that phagocytosis is thereby secondarily depressed. Future studies will investigate this hypothesis.

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