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Failure of Immune Sera to Enhance Significantly Phagocytosis of *Staphylococcus aureus*: Nonspecific Adsorption of Phagocytosis-Promoting Factors

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Serum from rabbits immunized with either heat-killed or live nonencapsulated *Staphylococcus aureus* failed further to enhance phagocytosis and intracellular killing of the homologous organism by either normal rabbit polymorphonuclear leukocytes or monocytes, when compared with normal rabbit serum. These immune sera did, however, show an increase in agglutinating and precipitating antibody level. Adsorption of normal human serum with some gram-positive and gram-negative bacteria, yeast, and some inert particles significantly reduced the phagocytosis-promoting factors of the serum. It would seem, then, that nonencapsulated *S. aureus* differs from other pathogenic bacteria in that the humoral antibacterial factors promoting its phagocytosis and intracellular killing are not significantly enhanced by infection or immunization.

It has long been known (4, 30) that specific immune sera are able to promote phagocytosis, at a much greater rate than normal sera, by combining with specific receptor sites on the surface of the antigenic particle (16, 17). Circulating specific antibody plays an important role in the phagocytosis of most bacteria, thereby protecting the host from the invading organisms. *Staphylococcus aureus* occupies a complicated and interesting position among these pathogenic bacteria. A few strains of *S. aureus* (Smith-type strains) have capsules and behave similarly to other pyogenic bactria, such as pneumococci, in that they resist phagocytosis in normal serum, whereas specific antibody promotes phagocytosis and killing. However, the great majority of pathogenic *S. aureus* strains, under normal conditions, do not show demonstrable capsules. Such common strains are readily phagocytized by leukocytes in the presence of normal serum and are killed slowly and incompletely (8, 22-24).

In the present study, attempts to augment the phagocytosis-promoting factors of serum by immunizing rabbits with heat-killed or live nonencapsulated *S. aureus* have been unsuccessful. It also has been found that the phagocytosis-promoting factors are not specific and can be removed nonspecifically from "normal" human serum by a variety of bacteria and inert materials.

MATERIALS AND METHODS

Bacteria. The following bacterial strains were used: *S. aureus* strains 18Z (the characteristics of this strain have been described previously; 8); Wood 46 (lacking protein A on the cell surface) and Cowan 1 (with excess protein A; 3); 3528, H, and Copenhagen (with α- and β- or both configurations of cell wall teichoic acids, respectively; 18); *S. albus* p77 (8); *Bacillus subtilis* and *Escherichia coli*, wild type.

Yeast. Dried bakers' yeast (Fleischmann's active dry yeast, Standard Brands Inc.) was used.

Suspensions of viable *S. aureus* strains used in the test system and for immunization were made from 18-hr-old cultures in Trypticase Soy Broth (BBL) and washed twice with saline. These then were heated at 80 C for 60 min (*B. subtilis* was autoclaved), washed three times with saline, and refrigerated. Heat-killed *S. aureus* used for immunization was prepared in the same manner but with the addition of 0.25% phenol. Dried yeast was suspended in saline and treated as mentioned above. All antigens were washed once more before using for adsorption.

Inert particles. The following inert materials were used for adsorption: polystyrene latex particles (Latex 0.81, Difco) washed three to four times with saline or dialyzed against distilled water for 30 hr; carbon black pelletized (Arthur H. Thomas Co., Philadelphia), silicic acid (Bio-SILBH, 100 to 200 mesh, Bio-RRD Lab., Richmond, Calif.), both washed three to four times with saline; bentonite (Fisher, U.S.P.) washed as described by Yotis and Ortiz (31); and Ballotini beads (Superbrite glass beads, type 100, Minnesota

Mining and Manufacturing Co., St. Paul) with an average diameter of about 0.2 mm, cleaned with nitric acid, washed several times with water, bicarbonate, and then distilled water.

Adsorption of serum. A 1-ml amount of 1:2 diluted normal human serum was added to a given amount of packed material as stated in experimental results. This was then mixed, rotated at 37 C for 60 min, and centrifuged; the supernatant was added to a new tube of the adsorbing material. This process was repeated for a total of two adsorptions (one with live *S. aureus*), and then the supernatant was filtered through a membrane filter (0.45 μm; Millipore Corp., Bedford, Mass.). A sample of serum which did not contain adsorbing materials was processed in an identical manner and used as a control. Adsorption of serum with live or heat-killed bacteria had similar results. Agglutination titers of hyperimmune rabbit and normal human sera were reduced only two- to fourfold after double adsorption.

Immunization of rabbits. (i) A dose of 10^9 live *S. aureus* was injected intradermally into each side of the rabbits once a week for 5 weeks. The rabbits were bled about 10 days after the last injection. (ii) Cell suspensions of heat-killed *S. aureus* containing 10^{10} to 5×10^{10} bacteria/ml were used. A 0.5-ml amount of the antigen was injected intravenously in the first week, and then 1.0 ml, by the same route, was injected at weekly intervals for 3 additional weeks. The rabbits were bled 1 week after the last injection.

Agglutination. The Boger et al. (1) and Lenhart et al. (12) method was used for titration of rabbit antistaphylococcal serum.

Agar-gel precipitation. Ouchterlony plates, either prepared and used as described by Haukenes and Oeding (6) and Oeding et al. (19), or I.D. discs obtained from Cappel Lab (Downington, Pa.) were used. Rabbit antihuman immunoglobulin (Ig)G, IgM, and IgA serum of Cappel Lab and *S. aureus* cell wall teichoic acid preparations (21) were used for precipitin lines with adsorbed and unadsorbed normal human serum or normal and immune rabbit serum, respectively.

Leukocytes. The rabbit exudate monocytes were induced by the intraperitoneal injection of mineral oil, as described previously (24). Rabbit exudate polymorphonuclear leukocytes were induced by the intraperitoneal injection of glycogen (20), and human blood leukocytes were separated by the dextran sedimentation procedure (20, 23). The leukocytes were washed twice with Hanks balanced salt solution.

Experimental procedures. Quantitative viable counts of staphylococci in suspensions of leukocytes and various sera were determined as follows. Washed leukocytes (10^7 to 2×10^7) were placed in a tube, serum was added to a final concentration of 5 or 10%, washed staphylococci were added in a ratio of one coccus per leukocyte, and the total volume was brought up to 3 ml with Hanks solution. The *p*H was maintained at about 7.4. The tubes were placed on a roller drum in an incubator at 37 C. Counts of viable, total, extracellular, and cell-bound staphylococci and of leukocytes were determined by the method described previously (23) with minor variations in the amount of sampling. Although in all experiments all above mentioned counts were performed, only those counts which gave sufficient information on phagocytosis and killing (cell-bound and total) are shown in the graphs. Other counts (extracellular and viable leukocytes) were in agreement with those counts given.

In determining the per cent phagocytosis in stained preparations, leukocytes, sera, and *S. aureus* were the same as used in the above procedure and the preparations were made at the same time. Washed leukocytes (3×10^6) in suspension, containing 5 to 10% serum, were placed in Porter flasks containing cover slips. The flasks were incubated at 37 C for 30 min to allow the leukocytes to adhere to the glass. Staphylococci were added in a ratio of 25 staphylococci per leukocyte and incubated an additional 30 min to allow phagocytosis. The cover slips were removed, washed in Hanks solution, and stained with Wright's stain. Quantitative counts of 200 to 300 leukocytes were made to determine the number of leukocytes containing staphylococci.

RESULTS

Normal and immune rabbit serum. The intracellular survival of *S. aureus* within normal rabbit peritoneal monocytes and polymorphonuclear leukocytes was studied in the presence of normal and immune rabbit serum. Rabbits were immunized with heat-killed or live nonencapsulated *S. aureus* strains 18Z, 3528, H, Copenhagen, Wood 46, and Cowan I, as described above. No significant difference was noted which could be attributed to use of live or heat-killed antigen.

Agglutination titers ranged from 200 to 400 in the normal rabbits to 1,600 to 5,200 in the immunized rabbits, depending on the strain used for immunization. Immune rabbit sera also gave precipitin lines in Ouchterlony plates with 10 μg of the corresponding *S. aureus* cell wall teichoic acids, whereas none of the normal rabbit sera showed precipitin lines with any of the teichoic acids tested.

Immune and normal rabbit sera were obtained at the same time. Both types of sera were either used fresh or were frozen at -20 C for comparison later. The following figures show examples comparing normal and immune rabbit serum, by using variations in types of cells, test organisms with their homologous antisera, and conditions under which sera were kept. Figure 1A represents one experiment on phagocytosis and killing of *S. aureus* Copenhagen in normal rabbit monocytes in the presence of fresh normal rabbit serum or fresh Copenhagen antiserum. Figure 1B shows the same type of experiment with 18Z as test organism and normal rabbit peritoneal polymorphonuclear leukocytes in the presence of normal rabbit serum and 18Z antiserum. In this last case, both sera were kept at -20 C for a few days before use.

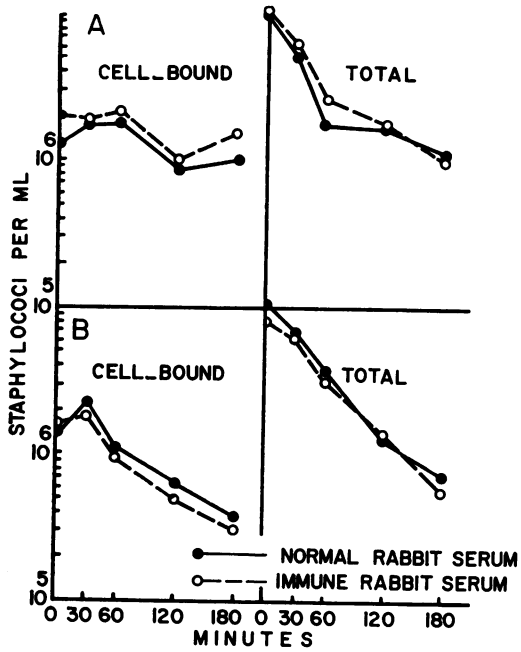

FIG. 1. *Cell-bound and total viable counts of S. aureus are shown from a representative experiment on the intracellular fate of S. aureus, comparing the effects of normal and homologous immune serum from rabbits. The leukocytes used were (A) monocytes and (B) polymorphonuclear leukocytes from normal rabbits.*

In the following figures, results from several of the same type of experiments were combined. In Fig. 2A, the data represented by the mean values were derived from 14 experiments comparing the use of normal rabbit sera with sera from rabbits immunized with heat-killed *S. aureus* 18Z, 3528, H, Copenhagen, Wood 46, and Cowan I, with the homologous *S. aureus* as test organism and normal rabbit monocytes. Figure 2B shows the mean values derived from 10 experiments, again comparing the use of normal rabbit sera with sera from rabbits immunized with heat-killed *S. aureus* 18Z, 3528, H, Wood 46, and Cowan I and homologous test organisms, but using normal rabbit polymorphonuclear leukocytes rather than normal rabbit monocytes. It may be seen in Fig. 1 and 2 that phagocytosis and intracellular killing of staphylococci do not differ significantly in the presence of normal or immune rabbit serum within either rabbit monocytes or polymorphonuclear leukocytes.

Leukocytes and sera used in the above experiments were also used to determine the per cent phagocytosis in stained preparations as described above. The per cent of both types of leukocytes which contained bacteria was similar with either immune or normal rabbit serum. As results with fresh or frozen sera did not differ significantly from each other in either of the above methods, results were combined.

Table 1 presents statistical analyses of variance of data from experiments comparing the use of normal and immune rabbit serum under a variety of conditions, including use of the two cell types, different immunizing strains, and homologous and heterologous test organisms, in both viable counts and stained preparations. It can be seen that serum from normal and immune rabbits promote phagocytosis and intracellular killing of non-encapsulated staphylococci at the same rate either in rabbit monocytes or polymorphonuclear leukocytes under any of the different conditions mentioned above.

Adsorption of normal human serum with gram-positive bacteria. Since specific immunization did not appreciably alter the effect on phagocytosis and killing, it was decided to study the more normally occurring factors which are found in healthy adult humans.

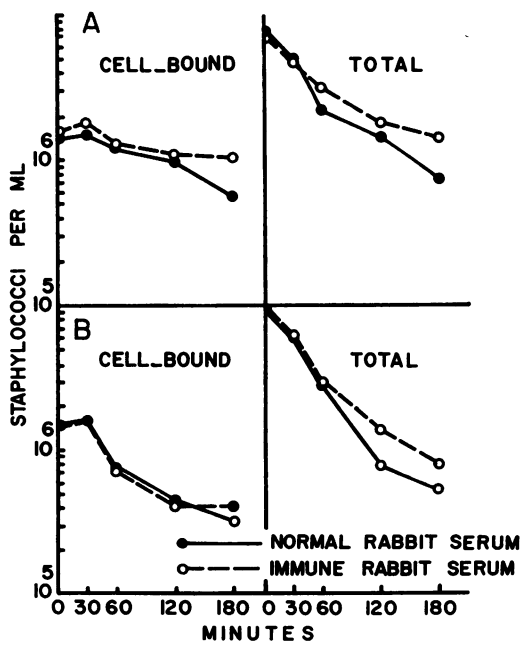

FIG. 2. *Mean cell-bound and total viable counts of S. aureus are shown from several experiments on the intracellular fate of S. aureus, comparing the effect of normal and homologous immune serum from rabbits. (A) Means of 14 experiments with normal rabbit monocytes and (B) means of 10 experiments with normal rabbit polymorphonuclear leukocytes. The S. aureus strains used for immunization and for determining the intracellular fate were 3528, H, Copenhagen, Wood 46, Cowan 1, and 18Z.*

TABLE. 1 *Statistical analyses of variance comparing the effect of normal with immune rabbit sera*

Normal rabbit peritoneal leukocytes	Sera from rabbit immunized with *S. aureus*	*S. aureus* used for immunization and for test	No. of tests	Statistical analyses of variance between normal and immune rabbit sera		
				Leukocytes in suspension (Viable count)		Leukocytes on glass (Stained)
				Intracellular at 30 min	Total at 180 min	30 min after phagocytosis
PMN leukocytes	Heat-killed: Wood 46, Cowan 1, 18Z, 3528 and H	Homologous	10	$P > 0.5$	$P > 0.5$	$P > 0.5$
PMN leukocytes	Heat-killed: 3528, H and Wood 46	Heterologous	7	$P > 0.5$	$P > 0.25$	
PMN leukocytes	Viable: 3528 and H	Homologous and heterologous	6	$P > 0.25$	$P > 0.25$	
Monocytes	Heat-killed: Wood 46, Cowan 1, Copenhagen, 18Z, 3528, and H	Homologous	14	$P > 0.5$	$P = 0.25$	$P > 0.5$
Monocytes	Heat-killed: H	Heterologous	2	$P > 0.5$	$P = 0.25$	

A 1-ml amount of 1:2 diluted normal human serum was adsorbed twice with 10^{11} heat-killed *S. aureus* or once with live *S. aureus*, as described above.

Adsorption of fresh human serum with *S. aureus* removed those factors responsible for phagocytosis and killing of homologous organisms. Heating of serum at 56 C for 30 min reduced the phagocytosis and intracellular killing of *S. aureus* (14, 24). When heated human serum was used for adsorption, little more reduction of phagocytosis and killing was observed when compared to the heated unadsorbed serum. For this reason, all other adsorptions were done with unheated serum.

Other gram-positive organisms were used for adsorption and these sera were tested with *S. aureus*. Figure 3 represents the mean values of experiments on blood leukocytes and sera from six normal humans, by using the method for viable counts of bacteria. Sera were adsorbed twice with about 10^{11} packed heat-killed *S. aureus* 3528, *S. albus* p77 or about 2×10^{10} packed heat-killed *B. subtilis*. *S. aureus* 3528 was used as the test organism. The cell-bound counts show that, with unadsorbed serum, there was phagocytosis for the first 30 min followed by phagocytosis and reduction in viable count. In comparison, with adsorbed sera the phagocytosis was slow with little or no killing. In the total viable counts, again with sera adsorbed with any

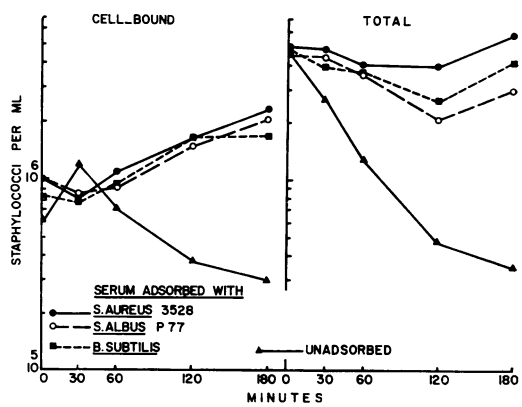

FIG. 3. *Mean cell-bound and total viable counts of S. aureus 3528. Comparison of the effect of normal human serum on the fate of the bacteria with that of the same serum adsorbed with either heat-killed S. aureus 3528, S. albus, or B. subtilis, by using autologous human blood leukocytes.*

one of these organisms, the rate of killing was equally reduced, as compared to the unadsorbed sera ($P < 0.005$).

Adsorption of normal human serum with gram-negative bacteria and yeast. When human serum was adsorbed twice with 10^{11} packed heat-killed *Escherichia coli* wild type, or 10^{10} yeast, results were comparable to adsorption with the above gram-positive organisms. Figure 4 shows the

mean values of three to five experiments by using the above two organisms and *S. aureus* 18Z for adsorption of serum and with autologous leukocytes and *S. aureus* 18Z in the test systems. It may be seen that adsorption with gram-negative bacteria and yeast give results similar to adsorption with the gram-positive bacteria seen in Fig. 3 ($P < 0.005$).

Adsorption of normal human serum with inert particles. The above experiments indicated that adsorption of phagocytosis-promoting factors for *S. aureus* in serum was not specific and that adsorption with other bacteria and yeast gave comparable results. In view of the above observations, adsorption with inert particles seemed to be the next logical step. Normal human sera (1 ml of 1:2 dilution), when adsorbed twice with 10^7 to 10^{10} polystyrene latex particles, were not significantly different from unadsorbed sera in their effect on the interaction between leukocytes and staphylococci. However, when 10^{11} particles were used for adsorption, reduced phagocytosis and killing did occur. Pelletized carbon black particles (100 mg), silicic acid particles (300 mg), and bentonite (20 mg) also affected the phagocytosis-promoting factors of human serum, although Ballotini beads (0.5 to 1 g) did not show any effect. Figure 5 shows the mean values of two to five experiments with each adsorbing material. Note that a significant reduction in phagocytosis and intracellular killing of *S. aureus* 18Z occurred when serum was adsorbed twice with 10^{11} polystyrene particles, 100 mg of carbon black, 300 mg of silicic acid, or 20 mg of bentonite, but not with 1 g of Ballotini beads ($P < 0.005$). However, this reduction was not as great as

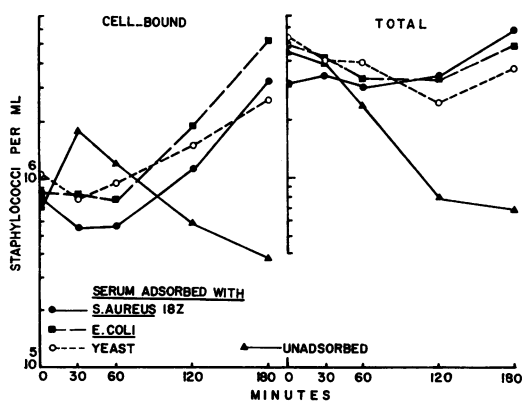

FIG. 4. *Mean cell-bound and total viable counts of S. aureus 18Z. Comparison of the effects of normal human serum on the fate of the bacteria with that of the same serum adsorbed with heat-killed S. aureus 18Z, E. coli, or yeast, by using autologous human blood leukocytes.*

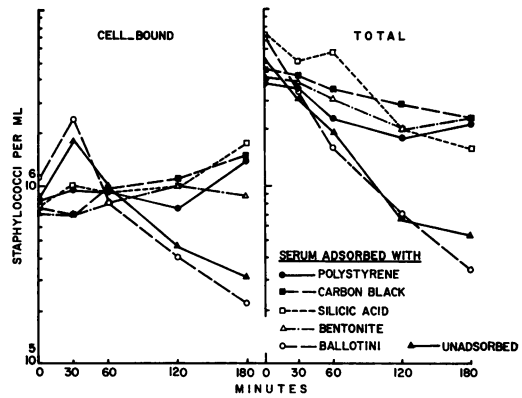

FIG. 5. *Mean cell-bound and total viable counts of S. aureus 18Z. Comparison of the effects of normal human serum on the fate of the bacteria with that of the same serum adsorbed with polystyrene, carbon, silicic acid, bentonite, or Ballotini beads, by using autologous human blood leukocytes.*

when bacteria and yeast were used for adsorption (Fig. 3, 4). In experiments using sheep red blood cells for adsorption, inhibition of phagocytosis also was observed.

DISCUSSION

Infection or experimental immunization with pathogenic bacteria characteristically elicits specific humoral antibacterial antibodies which promote phagocytosis. This fact has been known for more than seventy years (4, 30). These specific antibacterial antibodies combine with receptor sites in the bacterial surface, often fix complement, and thereby contribute to a bacterial surface upon which the surface membranes of polymorphonuclear leukocytes and macrophages can spread and envelop the bacterial cells in phagocytic vacuoles. *S. aureus*, and possibly virulent mycobacteria, would seem to be quite exceptional, in that specific antibacterial antibodies in the presence of heat-labile factors do not significantly enhance phagocytosis.

In the present study, the levels of agglutinating and precipitating antibody in normal rabbits were increased significantly by injection of heat-killed or live strains of a variety of widely used nonencapsulated *S. aureus*. The increased antibody titer was measured either by agglutination of living staphylococci or by precipitin lines formed by the antisera and *S. aureus* cell wall teichoic acid in double-diffusion agar plates. These immune rabbit sera, however, when tested with either normal rabbit peritoneal polymorphonuclear leukocytes or monocytes, failed further to enhance phagocytosis and intracellular killing of

homologous nonencapsulated *S. aureus* when compared to normal rabbit sera.

These experimental results are supported by others who used different approaches to this problem. Utilizing isolated perfused rabbit liver and rabbit serum, Koenig et al. (9) reported that, when serum from rabbits immunized with wild-type staphylococci was used, no significant change occurred in the hepatic removal of the homologous bacteria when compared to normal rabbit serum. Thorbecke and Benacerraf (27) showed that the clearance rates for *S. aureus* were similar in germ-free and control mice, and Downey and Pisano (5) found that polymorphonuclear leukocytes from germ-free rats or guinea pigs were not significantly different in the rate of phagocytosis of *S. aureus* from conventional animals. Thus, acquired specific antibacterial antibody appears to play little or no role in phagocytosis of nonencapsulated *S. aureus*. Cohen et al. (2) reported that sera from both nonimmunized germ-free and conventional mice have antibody against staphylococci.

Shayegani et al. (23, 24) reported that blood leukocytes or monocytes and autologous serum from immunized rabbits are not significantly different from those of normal rabbits in their effect on nonencapsulated *S. aureus*. Recently, Winston and Berry (29) showed that, although mice vaccinated with ribosomal extracts of *S. aureus* in Freund's complete adjuvant are protected against the challenge organisms, injection of heat-killed *S. aureus* failed to protect the mice against lethal doses of the bacteria. There are many reports on the phagocytosis-promoting effect of immune sera or protection of immunized animals from challenge organisms, but usually the capsulated Smith strain of *S. aureus* has been used in these studies (9).

The second part of this report shows that the phagocytosis-promoting factors of normal human serum for *S. aureus* could be removed, not only by adsorption with *S. aureus* but also with other unrelated bacteria and yeast. Most inert particles tested also showed the same effect but to a lesser extent. These results indicate the nonspecificity of the phagocytosis-promoting factors for nonencapsulated *S. aureus*. Lack of specificity also was reported by Hirsch and Strauss (7), who showed that by adsorbing normal rabbit serum with *S. albus*, the heat-labile serum opsonin was removed for salmonella as well as for the homologous bacteria. Smith and Wood (26) reported that adsorption of normal rat serum with *E. coli* removed the heat-labile opsonins for pneumococci, but that adsorption with polystyrene particles (0.184 μm diameter), did not, although in the present report a sufficient amount of polystyrene (0.81 μm diameter) did reduce the phagocytosis and killing of *S. aureus*. In contrast, specificity was suggested by Koenig et al. (9) who found that adsorption of normal serum with *S. aureus* reduced the hepatic uptake of *S. aureus* but not adsorption with *S. albus*.

Sera adsorbed with bacteria, yeast, and inert particles showed precipitin lines with rabbit anti-human IgG, IgM, and IgA serum similar to those of unadsorbed sera, indicating that the phagocytosis-promoting factors did not appear to be in any of these three immunoglobulin fractions. This is similar to the results of Hirsch and Strauss (7) who found that the heat-labile opsonin is not gamma globulin.

It has been known for a long time that heat-labile factors of serum promote the phagocytosis of various bacteria (30), and recently their effect on the phagocytosis and intracellular killing of *S. aureus* was reported (14, 24). These factors could be related to complement. When serum was adsorbed at 0 C, which tends to minimize loss of complement, very little difference was noted in phagocytosis and killing or in hemolytic complement titers [method of Lange (10)] when compared with the same serum adsorbed at 37 C. Some reduction in complement titer did occur (from $\frac{1}{16}$ to $\frac{1}{2}$) due to adsorption; however, it has not been determined whether this reduction was large enough to account for the reduction in phagocytosis-promoting factors and killing.

These factors, which are not augmented by immunization and which can be removed nonspecifically by a variety of materials, should be more fully characterized.

The principal purpose of our studies has been to determine what immunological means, if any, can significantly increase antibacterial resistance to ordinary, nonencapsulated *S. aureus*. Circulating antibodies against staphylococcal leukocidin have been considered in an earlier publication (15).

The present study of the phagocytosis-promoting action of the sera of normal and immunized rabbits offers little or no promise of advantage from immunization. Although it is possible that the antiteichoic acid or other heat-stable antibodies elicited by immunization may have of themselves some slight phagocytosis-promoting effect, in whole fresh serum this effect, if it exists, is lost in the greater effect of heat-labile components.

In human subjects, the phagocytosis-promoting action of heat-stable components and the complementing action of heat-labile components were clearly brought out by Wright and Douglas in 1903 (30) in the study in which the term *opsonin* was first used. Unfortunately, these healthy in-

vestigators used each other's sera and neglected to include the sera of infected or immunized subjects. Eight years previously the classic picture of the evocation of antibacterial phagocytosis-promoting antibodies by immunization had been set forth in the study of immunity against hemolytic streptococci by Denys and Leclef (4). The tacit assumption that effective antibacterial antibodies against ordinary staphylococci could be similarly evoked by vaccination has only rarely been challenged since. In consequence, tens of thousands of patients have been subjected to vaccination with autogenous or stock staphylococcal vaccines, with ambiguous and usually unsatisfactory results. A notable exception is the work of D. T. Smith (25), who has seen more deeply and used vaccines in relation to allergy against staphylococci.

Very recently investigators at the University of Minnesota have carried out analytical studies of great elegance in patients with subacute bacterial endocarditis (SBE; 11) and chronic osteomyelitis (28). Heat-stable gamma-G components of considerable phagocytosis-promoting efficacy were demonstrated in the sera of patients with SBE (11). In contrast, the heat-stable gamma-G phagocytosis-promoting components in the sera of patients with chronic staphylococcal osteomyelitis were found to be relatively weak.

However, in both of these clinical conditions, the heat-labile components of the fresh sera appeared to play the major opsonic role. "Heat-labile factors in fresh normal sera were more effective opsonins than heat-stable factors. Indeed there was little evidence for even an additive effect of heat-labile and heat-stable opsonins in normal sera as opsonin for *Strep. viridans* and *Staph. aureus*" (11). "Opsonic activities of both 7*S* and 19*S* fractions of heat-inactivated sera were studied in sera from osteomyelitis patients and control infections. When phagocytosis-promoting properties were not detectable in such fractions, opsonic capacity could be effectively restored by adding fresh serum devoid of antibacterial antibodies but possessing complement activity. Opsonic capacity of serum fractions then depended on the marked facilitative effect of heat-labile factors" (28).

From all of the above considerations in rabbits and in patients, we conclude that there is little promise of significantly improving phagocytic function against ordinary *S. aureus* by vaccination for the purpose of evoking antibacterial antibodies. The experience of D. T. Smith (25) and our own studies, however, do offer promise of benefit from elicitation of cell-mediated immunity and to this end the very high prevalence of delayed-type hypersensitivity in human populations (S. Mudd, J. H. Taubler, and A. G. Baker, J. Reticuloendothel. Soc., *in press*) offers a challenging opportunity.

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