

Extracellular and Bacterial Factors Influencing Staphylococcal Phagocytosis and Killing by Human Polymorphonuclear Leukocytes

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Extracellular and bacterial factors that influence the phagocytosis and killing of staphylococci by human polymorphonuclear leukocytes have been studied. *Staphylococcus epidermidis* strains were, in general, more rapidly phagocytized than were *S. aureus* strains. However, two strains of *S. epidermidis* had a very slow rate of ingestion. Although the rate of phagocytosis of *S. aureus* Wood 46 was greater than that of *S. aureus* 502A, the Wood 46 strain was more difficult to kill. Serum was essential for phagocytosis of both *S. aureus* and *S. epidermidis*. The opsonic titer of pooled serum was similar for *S. aureus* and *S. epidermidis*. In normal pooled serum, heat-labile factors were more important for effective phagocytosis than they were in immune serum. Although a saturation point for ingestion was reached, the percentage of ingested bacteria that remained alive within the leukocyte remained relatively fixed. Heat-killed and live staphylococci were ingested in a similar fashion. The rate of phagocytosis was greatly reduced at 41°C.

Phagocytosis and killing of staphylococci by polymorphonuclear (PMN) leukocytes serve as an important defense against infection. In one group of patients with a defect in this defense, chronic granulomatous disease, there is greatly increased susceptibility to frequent and severe staphylococcal infection (1, 19). These patients demonstrate the critical importance of an intact phagocytic system for adequate host defense. The cellular aspects of phagocytosis and bacterial killing have recently been reviewed (12, 20, 21).

Numerous extracellular factors that influence phagocytosis and bacterial killing have also been recognized. In general, opsonization is required for normal phagocytosis of bacteria. Two major systems in serum are opsonic for *Staphylococcus aureus*, a heat-labile system dependent upon complement (10, 22) and a heat-stable system mediated through immunoglobulin (11, 13, 18, 22). Opsonic requirements are less clearly established for *S. epidermidis* (10, 16). Other extracellular factors that might influence the phagocytic and bactericidal activities of leukocytes include the inoculum size (5), temperature (5, 8, 15), and certain drugs (3).

In addition to humoral and intrinsic leukocytic factors, certain components of the staphylococcus itself can influence the processes of phagocytosis and killing (7, 14). The state of

viability of the bacteria might likewise affect phagocytosis (6).

Before studying PMN leukocyte function and opsonic activity of serum in patients with recurrent staphylococcal infection, normal white cell phagocytosis and bacterial killing must be defined, and those extracellular and bacterial factors that might influence these processes must be standardized. It is the purpose of this study to investigate the influence of a number of these factors on normal PMN leukocyte ingestion and killing using a sensitive assay that measures both ingestion and intracellular survival.

MATERIALS AND METHODS

Bacterial strains. Two strains of *S. aureus*, 502A and Wood 46, and ten strains of *S. epidermidis*, six clinical isolates kindly provided by D. Williams and four commensal strains, were used. All the *S. epidermidis* strains belonged to biotype 1 (2).

Radioactive labeling. A 0.1-ml portion of an overnight culture of bacteria was inoculated into 10 ml of Mueller-Hinton broth (Difco, Detroit, Mich.) containing 0.02 mCi of [methyl-³H]thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear, Boston, Mass.). After 18 h of growth at 37°C, the bacteria were washed three times in phosphate-buffered saline (pH 7.4).

Leukocytes. From 10 to 20 ml of blood was drawn from healthy donors in a syringe containing 200 U of

heparin. The leukocytes were sedimented for 1 h in 6% dextran "70" (Cutter Laboratories, Berkeley, Calif.) in normal saline (10 ml of blood/3 ml of saline). The leukocyte-rich plasma was withdrawn and centrifuged at $160 \times g$ for 5 min. The resulting pellet was washed once in heparinized saline (10 U of heparin/10 ml of saline). Using a standard counting procedure, the final leukocyte suspension was made up to contain 10^7 PMN cells/ml of Hanks balanced salt solution with 1% gelatin.

Opsonins. Pooled human serum was diluted in Hanks balanced salt solution to the desired concentration. In one experiment sera from a patient with *S. aureus* and from a patient with *S. epidermidis* endocarditis were used. To test the need for heat-labile opsonic factors, in one experiment pooled human serum was heated at 56°C for 1 h before being added to the phagocytic mixture. When indicated, Hanks balanced salt solution or 3% bovine serum albumin was substituted for serum.

Phagocytic mixtures. Mixtures of 1 to 4 ml of leukocyte suspension, opsonin, and bacteria were prepared in plastic tubes (12 by 75 mm; Falcon, Oxnard, Calif.) in a volume ratio of 5:4:1. The desired bacterial concentration was determined spectrophotometrically. The final bacterial/PMN leukocyte ratios varied as indicated below. The mixtures were tumbled gently in a rotating rack (Fisher Roto-Rack; Fisher Scientific Co., Chicago, Ill.) at 37°C . In experiments designed to test the influence of temperature on the rate of phagocytosis, leukocytes, 10% pooled human serum, and bacteria were incubated separately at 37°C and 41°C for 1 h before preparing the final phagocytic mixtures.

Phagocytosis and bactericidal determinations. Phagocytosis and intracellular survival were determined using a method to be published in detail (J. Verhoef et al., submitted for publication). Duplicate 100- μl samples were taken from the phagocytic mixture with an Eppendorf pipette at determined intervals, placed in 3 ml of phosphate-buffered saline, (pH 7.4) in polypropylene vials (Bio-Vials; Beckman, Chicago, Ill.), and then centrifuged at $160 \times g$. The pellets were washed twice with phosphate-buffered saline. The final leukocyte pellet was disrupted with 2.5 ml of sterile distilled water. After vigorous mixing, samples were taken with a 5- μl Eppendorf pipette for plating in nutrient agar and determination of colony-forming units (CFU) after 18 h of incubation at 37°C . These CFU represented the surviving intracellular bacteria. The total ingested population of bacteria (alive and dead) was determined by centrifuging this final suspension at $1,600 \times g$ for 15 min and solubilizing the pellet in 2.5 ml of scintillation liquid (toluene-containing fluor-alloy TLA [Butyl-PBD and PBBO, Beckman] and 20% Biosolve-3 [Beckman]) and counting in a liquid scintillation counter (Beckman LS-250). To determine the total bacterial cpm (ingested and extracellular), duplicate 100- μl samples were taken at the end of the assay period, placed in 2.5 ml of water, and centrifuged at $1,600 \times g$ for 15 min. The pellets were suspended in scintillation liquid and counted. Immediately after the phagocytic mixtures were made up, duplicate 5- μl samples were taken

for plating and determination of the total CFU added to the mixture. For those experiments using bacteria/PMN cell ratios greater than 20:1, an appropriate dilution step was made before plating for both the total CFU and the intracellular surviving bacteria.

Calculations. The percentage of the total bacterial population that was phagocytized at a given sample time was calculated using the formula: % of bacteria ingested = (average of cpm in leukocyte pellet)/(average of cpm in total bacterial pellet) \times 100. The intracellular surviving bacterial population was determined using the formula: % of ingested bacteria which were alive = (% of total CFU alive in leukocyte pellet)/(% of total bacterial population ingested) \times 100, where the denominator was obtained from the formula used for calculating the percentage of bacteria ingested and the numerator was determined by the formula: (average of CFU in leukocyte pellet at sampling time)/(average of CFU at zero time) \times 100.

RESULTS

Effect of opsonins on phagocytosis of staphylococci. To determine which serum factors might influence PMN leukocyte uptake of staphylococci, phagocytosis was studied using five strains of *S. epidermidis* and two strains of *S. aureus* in pooled serum, heated serum, and bovine serum albumin. For both species of bacteria, heat-stable and heat-labile serum factors were opsonic. Of the five *S. epidermidis*, an average of 81% of the bacteria had been ingested in pooled serum after 20 min of incubation, compared with 38% in heated serum and 5% in bovine serum albumin. For each strain uptake was greater in pooled serum than heated serum; uptake in heated serum always exceeded that in bovine serum albumin. Similar findings were obtained with the *S. aureus* strains (Fig. 1). Some *S. epidermidis* strains were more difficult to phagocytize than others, and two strains were ingested at a slower rate than both *S. aureus* strains.

When pooled serum was diluted, phagocytosis decreased. Figure 2 illustrates this effect using *S. aureus* 502A. When the opsonic activity of serum from a patient with *S. epidermidis* endocarditis was compared with pooled serum, phagocytosis of a *S. epidermidis* strain was significantly enhanced. This enhancement was primarily related to an increase in heat-stable opsonic factors (Fig. 3). A similar effect was observed when serum from a patient with *S. aureus* endocarditis was used as an opsonic source for a *S. aureus* strain.

Effect of temperature on the phagocytosis of staphylococci. The phagocytosis of *S. aureus* Wood 46 and *S. epidermidis* strain 2 was tested at 37°C and 41°C using leukocytes, pooled serum, and bacteria that had been incu-

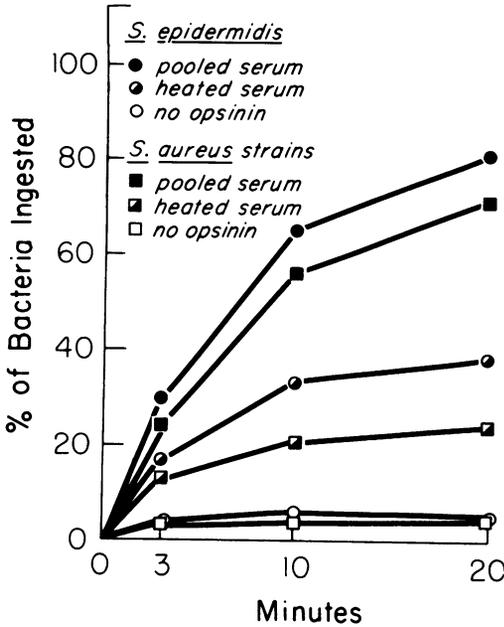


FIG. 1. Phagocytosis of *S. epidermidis* (average of five strains) in the presence of 8% pooled human serum (●), 8% heated pooled serum (◐) and 3 g% bovine serum albumin (○); *S. aureus* (average of two strains) in 8% pooled human serum (■), 8% heated pooled serum (◑) and 3 g% bovine serum albumin (□). Bacteria/PMN leukocyte ratio, 10:1.

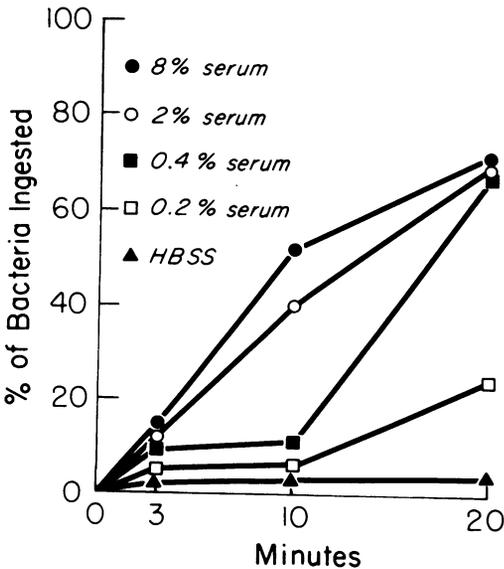


FIG. 2. Phagocytosis of *S. aureus* 502A in the presence of pooled human serum of different concentrations: 8% serum (●), 2% serum (○), 0.4% serum (■), 0.2% serum (□), and Hanks balanced salt solution (▲).

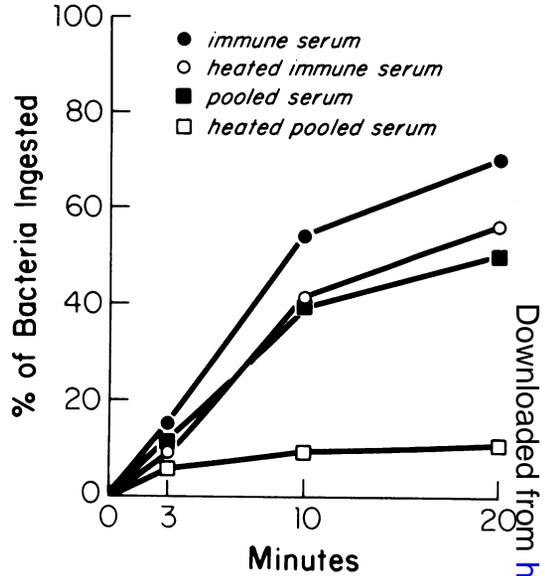


FIG. 3. Phagocytosis of *S. epidermidis* strain 2 in the presence of 8% immune serum (●), 8% heated immune serum (○), 8% pooled human serum (■), and 8% heated pooled serum (□). Bacteria/PMN ratio, 10:1.

bated separately for 1 h at these temperatures before performing the assay. Under these circumstances, phagocytosis of both bacterial strains was significantly reduced at 41°C. For *S. aureus* Wood 46, whereas 70% of the bacteria had been ingested at 20 min at 37°C, only 30% had been taken up by the leukocytes at 41°C (Fig. 4). Comparable results were obtained for the *S. epidermidis* strain.

Effect of inoculum size of staphylococci on phagocytosis and intracellular survival. Figure 5 shows the relationship between the inoculum size and the number of ingested bacteria after 10 min of incubation with PMN leukocytes. Initially, the number of phagocytized staphylococci increased in a linear fashion. For *S. aureus* 502A and *S. epidermidis* strain 1, saturation of ingestion was reached at an inoculum size of 5×10^7 to 10^8 CFU/ml. For *S. aureus* Wood 46, however, the number of ingested bacteria increased even when inoculum sizes exceeded 10^8 CFU/ml. The percentage of surviving intracellular *S. epidermidis* strain as a function of the inoculum size is illustrated in Fig. 6. Approximately 10% of the ingested bacterial population survived within the leukocytes independent of the inoculum size tested.

Phagocytosis of heat-killed versus live staphylococci. To study the effect of bacterial viability on phagocytosis, experiments were performed using living and heat-killed *S. au-*

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reus 502A and *S. epidermidis* strain 3 in different bacteria/PMN leukocyte ratios (2:1 and 50:1). In none of the experiments could a significant difference in phagocytosis be detected between live and dead bacteria (Fig. 7).

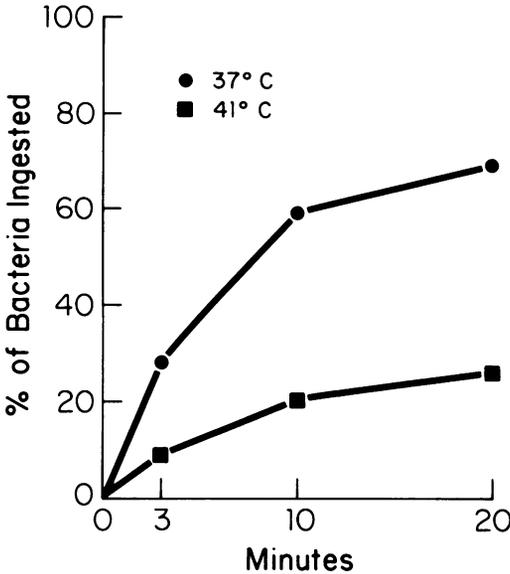


FIG. 4. Effect of incubation temperature, 37°C (●) versus 41°C (■), on the phagocytosis of *S. aureus* Wood 46. Bacteria/PMN leukocyte ratio, 10:1.

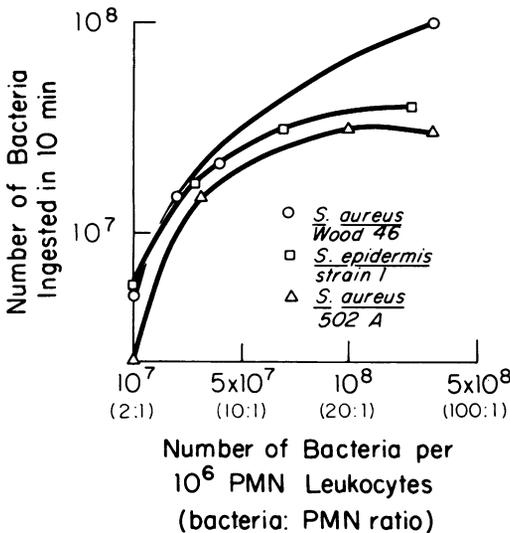


FIG. 5. Effect of bacteria/PMN leukocyte ratio on phagocytosis of *S. aureus* Wood 46 (○), *S. epidermidis* strain 1 (□), and *S. aureus* 502A (△). Samples were taken from the phagocytic mixtures after 10 min of incubation. Pooled human serum (8%) served as opsonin.

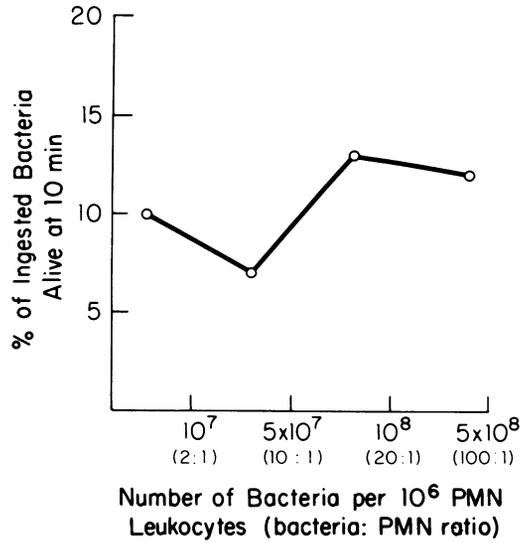


FIG. 6. Effect of bacteria/PMN leukocyte ratio on intracellular survival of *S. epidermidis* strain 1. Samples were taken from the phagocytic mixture after 10 min of incubation. Pooled human serum (8%) served as opsonin.

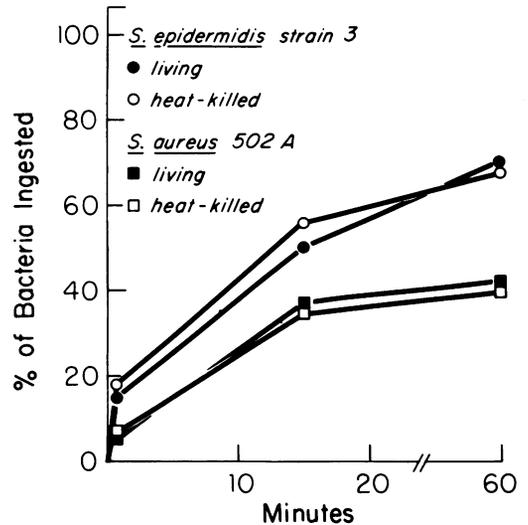


FIG. 7. Phagocytosis of *S. epidermidis* strain 3, living (●) and heat-killed (○), and of *S. aureus* 502A, living (■) and heat-killed (□), in the presence of 8% pooled human serum. Bacteria PMN leukocyte ratio, 50:1.

Phagocytosis and intracellular survival of *S. aureus* 502A and Wood 46. The rate of phagocytosis of *S. aureus* Wood 46 was greater than that of the 502A strain; however, the Wood 46 strain was more difficult to kill (Fig. 8). After 20 min of incubation, 85% of Wood 46 had been

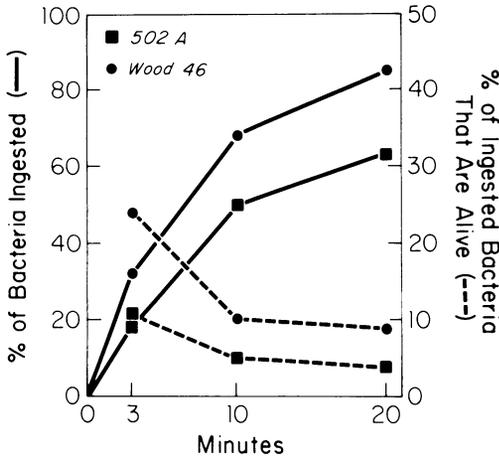


FIG. 8. Phagocytosis and intracellular survival of *S. aureus* Wood 46 (●) and 502A (■) in the presence of 8% pooled human serum. Bacteria/PMN leukocyte ratio, 10:1.

ingested compared with 63% of 502A. At the same time, 9% of the ingested Wood 46 population remained viable compared with 3% of 502A.

DISCUSSION

A sensitive assay system that measures both ingestion and intracellular survival of staphylococci in human PMN leukocytes was used to investigate a number of extracellular and bacterial factors that might influence these leukocyte processes. Heat-labile and heat-stable serum factors enhanced phagocytosis of the *S. aureus* strains as has been found by others (10, 11, 18, 22). This was also the case for all the *S. epidermidis* strains tested. This finding is in disagreement with the report of Miller and Beck, who found that no specific opsonic requirements were necessary for phagocytosis of *S. epidermidis* (16). In contrast to their results, no phagocytosis in bovine serum albumin and poor uptake in heated serum was found. Serum from a patient with *S. epidermidis* endocarditis was found to increase phagocytosis, as was true for one *S. aureus* strain tested. This suggests that antibodies as well as complement are needed for effective phagocytosis of *S. aureus* as well as *S. epidermidis*. Studies are being undertaken to determine the specific nature and sequence of activation of the heat-stable and heat-labile opsonic factors.

Leukocytes heated at 41°C for 1 h and then used in a phagocytosis assay carried out at 41°C had a significantly lower phagocytic capacity for staphylococci than controls (37°C). These findings are in contrast with those of Ellingson

and Clark, who found that elevated temperature increased the rate of phagocytosis (8), and with Mandell and Craig and Suter, who reported no difference in the ingestion rate at 37°C vs 41°C (5, 15). The preincubation of the PMN leukocytes for 1 h at 41°C in these experiments might explain the difference in the findings. Further studies are necessary to provide insight into the effect of varying temperatures on the processes of neutrophil phagocytosis and killing.

Experiments were done using different bacteria/PMN leukocyte ratios, heat-killed versus live bacteria, and different strains of *S. aureus* and *S. epidermidis* to study how various bacterial factors might influence phagocytosis and intracellular survival. Although a saturation point for ingestion was reached as the bacteria/PMN leukocyte ratio was increased, the percentage of ingested bacteria that remained alive within the leukocyte remained relatively fixed. This finding seemed to be consistent with the report of Craig and Suter, who suggested that the major limiting factor in PMN leukocyte handling of staphylococci was phagocytic and not bactericidal capacity (5).

No significant difference in the rate of phagocytosis of heat-killed versus live *S. aureus* and *S. epidermidis* was found. In a similar study of *Pseudomonas aeruginosa*, DeChatelet and co-workers found significantly reduced phagocytosis of heat-killed bacteria. They reported the same result with *S. epidermidis* (6). The reason for the discrepancy in the results is unclear, although it may be due to the higher ratios of bacteria/PMN leukocytes used by DeChatelet (greater than 100:1 as opposed to 2:1 to 50:1 used in these experiments).

A difference in phagocytosis and intracellular survival was found among the strains of staphylococci studied. *S. aureus* Wood 46 was phagocytized more rapidly than was the 502A strain and, in general, the *S. epidermidis* strains were more readily ingested than was *S. aureus* 502A. This might be due in part to the lack of protein A in the cell wall of the Wood 46 and *S. epidermidis* strains. This cell wall component, present in *S. aureus* 502A, is known to be antiphagocytic through its interaction with the Fc fragment of immunoglobulin G (7, 9). *S. aureus* Wood 46 appeared to survive the intracellular environment of PMN leukocytes more readily than 502A, a difference that Mandell also found and which he attributed to the high catalase content of Wood 46 (14).

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LITERATURE CITED

1. Baehner, R. L., D. G. Natan, and M. L. Karnovsky. 1968. Correction of metabolic deficiencies in the leukocytes of patients with chronic granulomatous disease. *J. Clin. Invest.* 49:865-870.
2. Baird-Parker, A. C. 1974. The basis for the present classification of staphylococci and micrococci. *Ann. N.Y. Acad. Sci.* 236:7-14.
3. Cline, M. J. 1974. Editorial: drugs and phagocytes. *N. Engl. J. Med.* 291:1187.
4. Cohn, Z. A., and S. I. Morse. 1960. Functional and metabolic properties of polymorphonuclear leukocytes. *J. Exp. Med.* 111:667-687.
5. Craig, C. P., and E. Suter. 1966. Extracellular factors influencing staphylocidal capacity of human polymorphonuclear leukocytes. *J. Immunol.* 97:287-296.
6. DeChatelet, L. R., D. Mullikin, P. S. Shirley, and C. E. McCall. 1974. Phagocytosis of live versus heat-killed bacteria by human polymorphonuclear leukocytes. *Infect. Immun.* 10:25-29.
7. Dossett, J. H., G. Kronvall, R. C. Williams, and P. G. Quie. 1969. Antiphagocytic effects of staphylococcal protein A. *J. Immunol.* 103:1405-1410.
8. Ellingson, H. V., and P. F. Clark. 1942. The influence of artificial fever on mechanisms of resistance. *J. Immunol.* 43:65-83.
9. Forsgren, A., and P. G. Quie. 1974. Effects of staphylococcal protein A on heat labile opsonins. *J. Immunol.* 112:1177-1180.
10. Forsgren, A., and P. G. Quie. 1974. Influence of the alternate complement pathway on opsonization of several bacterial species. *Infect. Immun.* 10:402-404.
11. Humphreys, D. W., L. J. Wheat, and A. White. 1974. Staphylococcal heat stable opsonins. *J. Lab. Clin. Med.* 84:122-128.
12. Karnovsky, M. L. 1975. Biochemical aspects of the function of polymorphonuclear leukocytes, p. 25-43. *In* J. A. Bellanti and D. H. Dayton (ed.), *The phagocytic cell in host resistance*. Raven Press, New York.
13. Laxdal, T., R. P. Messner, and R. C. Williams. 1968. Opsonic, agglutinating and complement fixing antibodies in patients with subacute bacterial endocarditis. *J. Lab. Clin. Med.* 71:638-653.
14. Mandell, G. L. 1973. Catalase, superoxide dismutase, and virulence of *Staphylococcus aureus*. *J. Clin. Invest.* 55:561-566.
15. Mandell, G. L. 1975. Effect of temperature on phagocytosis by human polymorphonuclear neutrophils. *Infect. Immun.* 12:221-223.
16. Miller, D. S., and S. Beck. 1975. Polymorphonuclear leukocyte phagocytosis: quantitation by a rapid radioactive method. *J. Lab. Clin. Med.* 86:344-348.
17. Mudd, S. E. 1970. A successful parasite: parasite-host interaction in infection by *Staphylococcus aureus*, p. 197-227. *In* S. E. Mudd (ed.), *Infectious agents and host reactions*. The W. B. Saunders Co., Philadelphia.
18. Nicherson, S. D., J. A. Kazmierowski, J. H. Dossett, R. C. Williams, and P. G. Quie. 1969. Studies on immune and normal opsonins during experimental infections in rabbits. *J. Immunol.* 102:1235-1241.
19. Quie, P. G., J. G. White, B. Holmes, and R. A. Good. 1967. *In vitro* bactericidal capacity of human polymorphonuclear leukocytes: diminished activity in chronic granulomatous disease in childhood. *J. Clin. Invest.* 49:668-679.
20. Stossel, T. P. 1974. Phagocytosis (first of three parts). *N. Engl. J. Med.* 290:717-723.
21. Stossel, T. P. 1974. Phagocytosis (second of three parts). *N. Engl. J. Med.* 290:774-780.
22. Wheat, L. J., D. W. Humphreys, and A. White. 1974. Opsonization of staphylococci by normal human sera: the role of antibody and heat-labile factors. *J. Lab. Clin. Med.* 83:73-78.