

## Evidence for the Importance of Bacterial Factors as Determinants of Susceptibility to Intraleukocytic Killing (40763)

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**Introduction.** Ingestion of bacteria by phagocytic cells has been a subject of continuing interest for years, and much has been learned about factors which influence bacterial engulfment. Quantitation of bacterial killing by phagocytic cells, on the other hand, has been somewhat obscured by the technical necessity of measuring the net results of ingestion and killing occurring as simultaneous events. Furthermore, direct comparison of the susceptibility of different bacteria to killing independent of opsonization has been limited, as it may relate to bacterial characteristics of importance in their behavior during infection.

In this general area, staphylococcal protein A, a cell wall component found in more than 90% of isolates of *Staphylococcus aureus* (1), is felt to represent a virulence factor for these organisms. It has been shown to impart resistance to phagocytic ingestion of the host *Staphylococcus* (2) presumably because of its ability to bind IgG at its Fc end, thereby competing with leukocyte Fc receptors for this part of the immunoglobulin molecule (2-4). Little direct evidence is available, however, on the effect of protein A on susceptibility of staphylococci to intraleukocytic killing. Recent studies by Peterson *et al.* (3) have suggested that an opsonized, protein A-containing *Staphylococcus*—though more resistant to ingestion—was more susceptible to intracellular killing than a protein A-deficient *Staphylococcus*.

A method previously developed (5) has allowed us to study quantitatively the process of bacterial killing by human polymorphonuclear leukocytes (PMN) distinct from their ingestive function. The assay is based on the observation that after termination of a finite period of ingestion, the rate of dis-

appearance of viable cell-associated bacteria is a measurement of phagocyte bactericidal activity. Application of this approach has revealed evidence for varying susceptibilities of different bacterial species to intraleukocytic killing independent of opsonic effects. These studies have also revealed that heat stable serum factors diminish killing of protein A-containing staphylococci by human PMN, when compared to a protein A-deficient *Staphylococcus aureus*. This observation demonstrates the importance of a bacterial virulence factor acting by protection of the interiorized organism from the phagocyte's killing powers as mediated via a component of normal human serum.

**Materials and methods. Medium.** Medium 199 (Gibco, Grand Island, N.Y.) with 0.1% gelatin (J. T. Baker Chemical Co., Phillipsburg, N.J.) was used throughout the experiments ("medium 199-gelatin").

**Bacteria.** Clinical isolates of *Staphylococcus epidermidis*, coagulase-positive *Staphylococcus aureus*, *Escherichia coli*, and *Enterobacter cloacae* were obtained from the clinical microbiology laboratory, Erie County Laboratory, Buffalo, New York. *Staphylococcus aureus* strains Wood 46 (protein A deficient) and Cowan I (protein A containing) were supplied through the courtesy of Dr. Paul G. Quie of the University of Minnesota School of Medicine. Overnight cultures of bacteria in nutrient broth (Difco, Detroit, Mich.) were twice washed by centrifugation in sterile phosphate-buffered saline, pH 7.0, and resuspended in Medium 199-gelatin solution to an optical density of 0.2 at 660 nm in a Coleman Junior II spectrophotometer. Bacterial concentrations as CFU/ml of medium 199-gelatin were determined for each bacterium by pour-plate colony counts of that standardized suspension.

**Leukocytes.** Venous blood from healthy

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individuals was obtained in heparinized vacutainer tubes. Leukocyte-rich plasma, obtained after sedimentation of red cells with 0.2 ml of 2.0% aqueous methyl cellulose solution per milliliter of blood, was centrifuged at 170g for 5 min and twice washed in medium 199–gelatin. Total and differential leukocyte counts were performed by standard techniques and the leukocyte suspension was adjusted to a final concentration of  $1 \times 10^7$  PMN/ml of medium 199–gelatin.

*Opsonins.* Blood from the same donor was collected in nonheparinized tubes, and serum collected after clotting. Preliminary experiments showed that the *E. coli* used was killed by fresh serum; the experimental format was, therefore, limited to use of inactivated serum as prepared by heating for 30 min in a 56° waterbath.

*Phagocytic mixtures.* Phagocytic mixtures were prepared in a volume of 1.5 ml in 12 × 75-mm capped polystyrene tubes (Falcon, Oxnard, Calif.) using medium 199–gelatin and the standardized suspensions of bacteria and leukocytes in volumes to supply  $3.8 \times 10^6$  PMN and a bacteria to PMN ratio of 50:1. Each experiment utilized duplicates done with and duplicates without addition of inactivated serum. All experiments except those exploring effects of different serum concentrations utilized serum at 10% v/v. Phagocytic mixtures were incubated with continuous mixing on an Eberbach rotating shaker for 10 min at room temperature (“ingestion phase”). Under these conditions replicate control experiments showed that 74% (SEM ± 1%) of the added protein A-positive *Staphylococcus aureus* became cell associated during the ingestion phase as determined by quantitation of bacteria remaining in the leukocyte-free supernatant of the phagocytic mixture at the end of the incubation period.

*Bactericidal assay.* Immediately after the 10-min ingestion phase the contents of each phagocytic reaction tube was divided into 0.2-ml aliquots which were transferred to sterile microtiter wells (Cooke Lab. Products, Alexandria, Va.). The plate was centrifuged at 170 g for 3 min and the supernatant fluid with non-cell-associated bacteria

decanted. The leukocyte pellets were twice washed with 0.2 ml medium 199–gelatin with separation by centrifugation at 170g for 3 min. The final white cell pellet was resuspended in the original volume of medium 199–gelatin. At this point one set of replicate wells was transferred individually into sterile distilled water for PMN lysis. An additional set of wells was allowed to incubate with gentle agitation at room temperature for 40 min (“killing phase”), and then these wells, too, were transferred to sterile distilled water for PMN lysis. Viable bacteria in each well were determined as colony-forming units (CFU) by duplicate plate counts (tryptic soy agar) of aliquots of appropriate distilled water dilutions of the original lysate.

*Controls.* Appropriate controls were included to show that within the operational time of these experiments the bacteria remained in lag phase. Further, no reduction in viable CFU of bacteria occurred following incubation of any of the bacteria used with the inactivated sera alone. Control studies showed that as performed here differential centrifugation removed 95% of the non-cell-associated bacteria, as defined by that proportion of added bacteria which could be removed by additional centrifugal separations.

*Data handling.* For each experiment this format provided 12 counts of viable, cell-associated, colony-forming units (CFU) for each initial and each final (after 40 min of killing) set of wells. On each set the highest and lowest values were discarded, and the percentage change in viable intracellular CFU was determined using means of the remaining 10 values. The bactericidal index (BI) is defined as the fractional change in bacterial CFU over 40 min/ml of original phagocytic mixture (containing  $2.5 \times 10^6$  PMN), and calculated as

$$\frac{\text{Mean CFU}_{\text{initial}} - \text{mean CFU}_{\text{after}}}{\text{Mean CFU}_{\text{initial}}}$$

For statistical analysis, all experiments were grouped according to the test organism and differences between the mean bactericidal indices for conditions with and without heated serum were evaluated

TABLE I. BACTERICIDAL ACTIVITY OF NORMAL HUMAN PMN AGAINST BACTERIA: VARIATIONS BETWEEN ORGANISMS AND THE EFFECT OF AUTOLOGOUS SERUM

Bacterium	N	Without serum		10% serum added	
		BI <sup>a</sup>	Initial CFU <sup>b</sup>	BI	Initial CFU
<i>Staphylococcus epidermidis</i>	4	0.32 ( $\pm 0.10$ )	2.40 ( $\pm 0.15$ )	0.44 ( $\pm 0.06$ )	2.22 ( $\pm 1.35$ )
<i>Escherichia coli</i>	4	0.15 ( $\pm 0.07$ )	4.03 ( $\pm 0.65$ )	0.26 ( $\pm 0.06$ )	3.80 ( $\pm 0.58$ )
<i>Enterobacter cloacae</i>	2	0.02 ( $\pm 0.02$ )	3.04 ( $\pm 0.43$ )	0.18 ( $\pm 0.15$ )	1.78 ( $\pm 0.61$ )

<sup>a</sup> Bacterial index (see text); mean  $\pm$  SEM.

<sup>b</sup> Cell-associated, bacterial, colony-forming units at beginning of period of digestion,  $\times 10^{-7}$  per  $2.5 \times 10^6$  PMN; mean  $\pm$  SEM.

statistically. Statistical significance was ascribed to *P* values less than 0.05.

**Results.** *Bactericidal indices for bacteria other than Staphylococcus aureus.* Average bactericidal indices of human PMN for *Staphylococcus epidermidis*, *Escherichia coli*, and *Enterobacter cloacae* are presented in Table I. Inspection of these data reveals a significant variation in the average BI of human PMN for the different bacteria in the absence of serum. Since each of the experiments (e.g., four for *E. coli*) involves replicate, simultaneous determinations of the BI for PMN from a single donor, the possibility was considered that intrinsic differences in killing rate existed as a function of cell donor. Inspection of the data with this question in mind, however, failed to reveal such a correlation.

The data in Table I also indicate that for these gram-negative and -positive organisms the addition of 10% autologous serum to the system uniformly increased the average BI by factors ranging from 1.4:1 to 8:1. Eight of the ten individual experiments involved showed an enhancement of BI by addition of serum. Because of the wide range of values, this observation just misses statistical significance when tested by the Student's *t* test ( $0.1 > P > 0.05$ ), but achieves significance at the 0.05 level by the Wilcoxon matched-pairs, signed, rank test ( $t = 8$ ).

Within limits, the bactericidal index has been found to be independent of the bacteria to PMN ratio in the phagocytic mixture. In 15 experiments utilizing PMN from

different donors and the same staphylococcal isolate, the BI was found to be independent of alterations in this ratio during the ingestion phase within the range of 40:1 to 100:1 (Matheisz, J. S. and Allen, J. C., unpublished data). The number of cell-associated bacteria following the ingestion phase correlates within limits with the bacteria-to-phagocyte ratio in the phagocytic mixture. In our experimental format the number of cell-associated bacteria at the beginning of the killing phase is subject to the influence of both ingestion and killing as they have occurred to the time of measurement. Inspection of the data provided in Table I reveals that comparable numbers of bacteria were PMN associated at the beginning of the killing phase, and fails to reveal any clear correlation between the number of bacteria at this time and the bactericidal index subsequently found. As further confirmation of this point, the experiments on *Staphylococcus aureus* shown here have revealed a highly reproducible BI (range 0.41 to 0.45) to be associated with almost a 50% difference in cell-associated CFU at the beginning of the killing phase ( $3.57$  to  $6.42 \times 10^7$  CFU per  $2.5 \times 10^6$  PMN).

*Bactericidal indices for Staphylococcus aureus.* Study of a random isolate of coagulase-positive *Staphylococcus aureus*, on the other hand, gave strikingly different results (Table II). All five experiments with this organism revealed an inhibition of intraphagocytic killing by the addition of 10% autologous serum, as signaled by a fall in average BI from 0.42 without serum to 0.10

TABLE II. BACTERICIDAL ACTIVITY OF NORMAL HUMAN PMN AGAINST COAGULASE-POSITIVE *STAPHYLOCOCCUS AUREUS*: STUDIES WITH PROTEIN A-SELECTIVE STRAINS

Staphylococcus strain	N	Mean bactericidal index	
		Without serum	10% serum added <sup>a</sup>
Random isolate	5	0.42 ( $\pm 0.08$ ) <sup>b</sup>	0.10 ( $\pm 0.05$ )
Cowan I (protein A)	4	0.45 ( $\pm 0.08$ )	0.29 ( $\pm 0.05$ )
Wood 46 (deficient)	5	0.12 ( $\pm 0.03$ )	0.20 ( $\pm 0.08$ )

<sup>a</sup> Autologous, heat inactivated.

<sup>b</sup> Standard error of the mean.

with serum. This change in means is highly significant by the Student's *t* test ( $t = 6.58$ ;  $P < 0.01$ ).

In an effort to establish a bacteriologic correlate with this serum inhibitory effect, attention was turned to protein A, a constituent of staphylococcal cell walls with an established relationship to phagocytosis. A strain of *Staphylococcus aureus* known to contain protein A (Cowan I; Ref. (2)) and one deficient in protein A (Wood 46; Ref. (3)) were studied in this system. The results as shown in table II reveal a striking correlation between serum inhibition of intraphagocytic killing and the presence of protein A. Studies with the Cowan I strain, known to contain protein A revealed a no-serum BI of 0.45 which was in close agreement with the no-serum BI determined for our random isolate *Staphylococcus aureus* (0.42). As also seen in studies with the random isolate, addition of 10% autologous serum reduced this BI to a statistically significant degree (BI = 0.29;  $t = 3.73$ ;  $P < 0.05$ ). Studies with the protein A-deficient, Wood 46 strain, on the other hand, revealed a statistically significant *increase* in BI in the presence of 10% autologous serum ( $t = 3.38$ ;  $P < 0.05$ ), as was seen in studies of other bacterial species (Table I).

As further confirmation of this effect, three replicate experiments seeking a dose-response effect were undertaken utilizing the protein A-containing *Staphylococcus aureus*. The averages of these experiments are shown in Table III and indicate an inhibition of the BI from a value of 0.41 without serum, to 0.34 with 5%, and 0.24 with 15% added autologous serum.

**Discussion.** The function of phagocytic cells leading to death of bacteria can be vi-

sualized as the summation of two major activities: interiorization of the bacteria followed by their killing. While a variety of approaches to quantitation of ingestion have been used, measurements of the killing function have been based primarily on modifications of the technique originally proposed by Maaloe (6). This technique involves the mixing of bacteria and phagocytes in an appropriate incubation milieu with the periodic measurement of the numbers of remaining viable bacteria in the entire reaction mixture and the numbers of viable bacteria associated with the phagocytic cells. It is clear, however, that since the bacterium can only be killed after it is interiorized, disappearance of viable bacteria from such mixtures actually depends not only upon their intracellular demise, but also upon the effectiveness of the uptake (i.e., ingestive) mechanism of the phagocytic cells. In addition, such systems have the inherent disadvantage that both the ingestive and digestive functions are going on simultaneously during the entire course of the experiment. This situation could obscure mild defects in digestive function, though it obviously detects gross ones (7). Furthermore, evidence has been presented that killing capacity of human granulocytes may be diminished by repetitive exposure to bacteria (5), so continuing

TABLE III. EFFECT OF DIFFERENT CONCENTRATIONS OF AUTOLOGOUS SERUM ON BI OF HUMAN PMN FOR PROTEIN A-CONTAINING *STAPHYLOCOCCUS AUREUS*

Serum concentration (%)	Mean BI ( $\pm$ SE)
None	0.41 ( $\pm 0.07$ )
5	0.34 ( $\pm 0.02$ )
15	0.24 ( $\pm 0.06$ )

phagocytosis may in and of itself depress killing capacity of phagocytic cells.

The importance of experimentally separating the ingestive and killing activities of phagocytic cells has been recognized by many investigators (8-11), and several experimental approaches to accomplish this have been proposed as modifications of Maaloe's original technique. Our approach utilizes a short exposure of these cells to a known quantity of bacteria, removal of the remaining bacteria not cell associated, and then determination of the disappearance of viable bacteria from the phagocytes during a measured period thereafter. This technique provides at least some dissociation of the ingestive and killing phases of phagocytic activity. It has been shown to correlate with other measures of bacterial killing by phagocytes, and has distinguished a defect of the killing as opposed to the ingestive function (5).

Phagocytosis of multiple bacterial species has been studied, and the general observation that heat-labile and heat-stable opsonins play a facilitating role in that process is established. While experimental approaches to study of phagocytic bactericidal function have been many, no direct, side-by-side comparison of the susceptibility of different bacteria to intraleukocytic killing independent of opsonic factors has appeared. While this situation is obviously not encountered *in vivo* during the course of bacterial infection, information so gained may aid in designation of bacterial components important in determining their fate during encounters with phagocytic cells (c.f., staphylococcal protein A). The data we have presented suggest that various bacteria have intrinsically different susceptibilities to the bactericidal powers of human PMN independent of opsonic effects. Furthermore, these differences appear large (about 20-fold between the highest and lowest bactericidal indices), and independent of the virulence of the organisms as judged from their role in human infections. Thus, while the gram-negative pathogens studied have low bactericidal indices compatible with relative resistance to intraleukocytic killing, the virulent *Staphylococcus aureus* shares with the rel-

atively benign *Staphylococcus epidermidis* a high index, indicating susceptibility to intraleukocytic killing. The possibility that the bactericidal index is related to characteristics of the cell wall correlating with its gram staining is raised by the data, though behavior of the Wood 46 *Staphylococcus aureus* appears against this interpretation.

If taken at face value, the low BIs noted for *E. coli* and *Enterobacter cloacae* (Table I) suggest they have a better chance for prolonged intracellular survival than does the *Staphylococcus aureus* with its high BI (Table II). How accurately the experimental design mimics the situation in actual human infection, however, cannot be determined. Thus, many things during the course of infection probably influence the ultimate relationship between phagocytic cells and ingested bacteria. In that situation, continuous ingestion is occurring and undoubtedly fatigue in PMN killing function occurs (5). Further, many host defense factors are in action *in vivo*, including alterations in nature of the milieu, changing ratios of bacteria to phagocytic cells, and the presence of factors—primarily complement—which may profoundly influence the relationship between bacterium and phagocytic cell.

It is important to reiterate that phagocytes from several different donors were utilized in each of the experiments, and no correlation between results and cell donor could be determined. Further, reproducibility of the technique is probably best witnessed by noting the bactericidal indices determined on three occasions for the *Staphylococcus aureus* (containing protein A): 0.41, 0.42, and 0.45. These experiments were done at intervals separated by weeks or months, and support the reliability of the measurements reported.

Though limitations in experimental design may preclude definitive conclusions in this area, data exist which indicate the presence of serum opsonins enhances both the ingestion and the killing of a number of different bacterial species by human phagocytic cells. The data developed here (Table I) support this conclusion relative to bacterial killing. The majority of human virulent, coagulase-positive *Staphylococ-*

*cus aureus*, on the other hand, have stood as a notable exception to this generalization. Thus, the presence of protein A has been clearly shown to impede the ingestion of its host *Staphylococcus* by human PMN in the presence of serum opsonins (3, 4), compared with strains lacking protein A. On the other hand, it is clear that serum opsonins provide enhancement of ingestion of protein A-containing strains, just as they do for other bacterial species. Our studies suggest a significant difference in the role of heat-stable serum opsonins on the intraleukocytic killing of protein A-containing staphylococci, both when compared with several other bacterial species and when compared with protein A-deficient staphylococci as well. Presence of these opsonins offers a significant protection to the *Staphylococcus* with protein A, as indicated by a fall in the bactericidal index when heat-inactivated serum is added. Peterson *et al.* (3) noted a greater resistance of protein A-deficient staphylococci to intraleukocytic killing in the presence of serum opsonins, when compared with a strain containing protein A, an effect ascribed by Mandell to the high catalase content of protein A-deficient staphylococci (11). Our data (Table II) are in agreement with these observations in the detection of a lower bactericidal index for the Wood 46 (deficient) compared to the Cowan I strain. The difference between the two was 9% on our scale of measurement, a number in good agreement with the 6% difference noted by Peterson *et al.* (3). Our data augment this observation in showing a striking enhancement of resistance of the protein A-containing *Staphylococcus* to intraleukocytic killing by the addition of heat-stable opsonins. This observation offers further evidence for the importance of bacterial factors in bacterial resistance to phagocytic attack.

A distinction between surface association and interiorization of bacteria by phagocytic cells has been suggested as an explanation for apparent differences between bacterial susceptibilities to intraleukocytic killing (12). Since our techniques study only cell-associated bacteria and since the enhancement of ingestion by serum opsonins

is a well-documented observation even with respect to the protein A-containing *Staphylococcus* (3), the antagonism of serum factors to leukocytic killing of the protein A-containing *Staphylococcus* cannot be explained by an alteration in bacterial interiorization alone. The mechanisms involved in this observation, however, remain conjectural. Our studies have only shown a correlation between the serum depression of intraphagocytic killing of *Staphylococcus aureus* and the presence of protein A in the organism; that protein A is the responsible factor has not been proven. Further, nature of the heat-stable serum factor(s) has not been demonstrated, though the affinity of protein A for immunoglobulin G suggests involvement of these molecules. It is of interest that Shayegani *et al.* (9) demonstrated that heated serum from rabbits immunized with staphylococci blocked phagocytic killing of those organisms, compared with heated serum from the same animals before immunization. The possibility that as a result of avidity or other characteristics, specific antibody against the *Staphylococcus* may provide selective influences on its interaction with phagocytic cells in contrast with the nonspecific immunoglobulin binding associated with protein A must be considered, and may be subjected to test using the observation reported here. It must be acknowledged that a direct effect of serum factors on the leukocyte, rather than the bacterium, cannot be excluded by our experimental design. Since autologous serum was used, and since the enhancing effect of serum on killing by leukocytes from the same donor differed with the various organisms, this is felt to be an unlikely explanation for our observations, however.

How alteration of a bacterium by serum factors can impede its susceptibility to intraleukocytic killing is as yet unclear. Possibly steric interference with the various intracellular factors leading to bacterial death is involved, or possibly alteration of the bacterial surface to a more resistant configuration occurs. Goldstein *et al.* (13) have demonstrated that exposure of PMN to an Fc-receptor stimulus triggers the intracellular biochemical events associated

with bacterial killing and lysis (generation of superoxide anion and release of lysosomal enzymes). Others (10, 14, 15) have proposed that the Fc fragment of IgG is a critical determinant to effective phagocytic ingestive and killing activities, as its presence on the bacterial surface triggers these activities via the leukocytic surface Fc receptor sites. It is most appealing that the competition for these Fc sites by staphylococcal protein A which is known to be important in impeding ingestion of such organisms by PMN may also play a role in muting the stimulation of intracellular mechanisms for bacterial killing. The role of this observation as an explanation for the facultatively intracellular existence of *Staphylococcus aureus* (16), and as an example of bacterial manipulation of host factors to enhancement of its own virulence, is a possibility of interest which remains to be explored.

*Summary.* Studies of intraleukocytic bacterial killing by a technique which allows dissociation of the killing from the ingestive function of human polymorphonuclear leukocytes have revealed differing susceptibilities of several bacterial species to killing in the absence of opsonization. While heat-stable opsonins enhance leukocytic killing of various bacteria, they were shown to impede killing of a human virulent *Staphylococcus aureus*, an effect correlated with the presence of protein A in a test strain. These observations indicate the importance of bacterial factors to the susceptibility of bacteria to intraleukocytic

killing, an effect mediated via heat-stable opsonins in the case of protein A-containing *Staphylococcus aureus*.

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