

## Enhanced Killing of Penicillin-Treated Gram-Positive Cocci by Human Granulocytes: Role of Bacterial Autolysins, Catalase, and Granulocyte Oxidative Pathways

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Staphylococci pretreated with subminimal inhibitory concentrations (subMIC) of cell-wall active antibiotics exhibit increased susceptibility to killing by human polymorphonuclear leukocytes (PMNs), even when phagosome information is impaired by the mold metabolite, cytochalasin B. To investigate the role of specific bacterial factors in the process, studies were carried out with organisms lacking catalase (streptococci) or cell-wall autolytic enzymes and compared to findings with *Staphylococcus aureus* 502A. Neutrophil factors were studied using inhibitors, oxygen radical scavengers, myeloperoxidase (MPO)-deficient PMNs, or PMNs from a patient with chronic granulomatous disease (CGD).

Documentation of the enhanced susceptibility of the streptococcal strains to killing by PMNs following subMIC penicillin pretreatment required the use of cytochalasin B. Enhancement of killing occurred independent of the presence or absence of bacterial autolysins or catalase. SubMIC penicillin pretreatment of *S. pneumoniae* R<sub>36</sub>A specifically promoted the susceptibility of these organisms to killing by myeloperoxidase (MPO)-mediated mechanisms (enhancement lost using MPO-deficient or azide-treated cells). Factors other than MPO or toxic oxygen products generated by the PMN respiratory burst are responsible for enhanced killing of penicillin-pretreated *S. aureus* 502A (enhancement preserved using MPO-deficient, azide-treated, or chronic granulomatous disease patient cells).

These studies define methods to study the interaction of antimicrobial agents and PMNs in the killing of microorganisms. They also demonstrate that penicillin treatment can change the susceptibility of gram-positive cocci to the action of specific PMN microbicidal mechanisms. The mechanism of the enhancement appears to be bacterial strain-dependent and not predictable by bacterial autolysin or catalase activity.

### INTRODUCTION

We have previously shown [1,2] that laboratory and clinical isolates of *S. aureus*

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pretreated with subminimal inhibitory concentrations (subMIC) of cell-wall active antibiotics (penicillin, vancomycin), but not ribosomally active drugs such as gentamicin, are more susceptible to killing by human polymorphonuclear leukocytes (PMNs). This increased susceptibility is present even when bacterial uptake and phagosome formation of the PMN are inhibited by cytochalasin B. In these experiments, all penicillin-treated bacteria which were ingested by or bound to normal or cytochalasin B-treated PMNs were killed as opposed to only a fraction of the untreated bacteria.

*S. aureus* are catalase-positive organisms which contain autolytic enzymes that might be triggered by penicillin [3]. In order to assess the role of bacterial catalase, as well as the role of autolysins in the enhanced killing of cell-wall active antibiotic-treated bacteria, experiments were performed with three well-characterized strains of streptococci and the results compared to similar experiments with *S. aureus* 502A. *Streptococcus pneumoniae* R<sub>36</sub>A, a rough "wild-type" pneumococcus, was chosen since it is catalase-negative and has an autolysin-defective mutant [4,5], and the absence of a capsule avoided alterations in phagocytosis and killing due to loss of capsular polysaccharide following antibiotic pretreatment. *Streptococcus sanguis* strain "Wicky" is an autolysin-defective Lancefield group H hemolytic streptococcus. It shares with the autolysin-defective R<sub>36</sub>A pneumococcus the feature of antibiotic tolerance; that is, penicillin G inhibits growth in low concentrations but does not cause lysis and produces only a minor loss in viability during pretreatment [6]. To examine the role of PMN oxidative mechanisms in the killing process, studies were performed with cells from patients with chronic granulomatous disease (CGD) and myeloperoxidase (MPO) deficiency as well as normal PMNs treated with sodium azide. Furthermore, a variety of oxygen reduction product scavengers were examined for their ability to modify killing of penicillin-pretreated *S. aureus* 502A.

Similar to the findings with staphylococci, penicillin pretreatment enhanced the killing of all three streptococcal strains by PMNs in the presence of cytochalasin B. The basis of this enhancement differed, however, with respect to mechanisms employed by the PMNs and the bacterial species studied. Oxidative mechanisms appear essential for enhanced killing of *S. pneumoniae*; non-oxidative mechanisms are more important in the killing of *S. aureus* and the autolysin-defective *S. sanguis* var. Wicky. These studies define an approach to investigating potentially important interactions between antibiotics and PMNs in the killing of different bacteria.

## MATERIALS AND METHODS

### Reagents

Cytochalasin B was purchased from Aldrich Chemical Co., Inc., Milwaukee, WI, dissolved in dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO) to a concentration of 2.5 mg/ml and frozen at  $-20^{\circ}\text{C}$ . It was used in the experimental suspensions at a final concentration of 5  $\mu\text{g}/\text{ml}$  and fresh aliquots were thawed and used daily.  $^{14}\text{C}$ -labeled mixed L-amino acids and Aquasol<sup>®</sup> for liquid scintillation counting were purchased from the New England Nuclear Co., Boston, MA. Trypticase soy agar (TSA) and broth (TSB) were purchased from Baltimore Biological Laboratories, Cockeysville, MD. Hank's balanced salt solution (HBSS) came from Flow Laboratories, Rockville, MD. 3 percent dextran in saline and penicillinase were purchased from Sigma Chemical Co. Penicillin G was obtained from E.R. Squibb and

Son, Inc., Princeton, NJ. 12 × 75 mm pyrex borosilicate tubes were purchased from Corning Glass Works, Corning, NY. Normal human serum was obtained from the clotted blood of healthy consenting volunteers, pooled, separated into aliquots, and stored at -70°C until used.

### *Microorganisms*

*Streptococcus pneumoniae*, strain R<sub>36</sub>A (wild type, Rockefeller University Laboratory stock) an autolysin-defective mutant derivative of the R<sub>36</sub>A strain [4,5] (Cwl), and the group H hemolytic *Streptococcus sanguis*, strain Wicky, were kindly supplied by Drs. Diane Horne and Alexander Tomasz of the Rockefeller University.

Stocks of each strain were frozen without aeration with 10 percent glycerol in C medium supplemented with 0.1 percent yeast extract [7] also supplied by Drs. Horne and Tomasz. All of the above organisms are catalase-negative. The coagulase and catalase-positive, penicillinase-negative *Staphylococcus aureus* H.R. Shinefield 502A, was used in a portion of the experiments [1]. The minimal inhibitory concentrations (MIC) of all organisms were determined by a previously described method [8]. The *S. pneumoniae* R<sub>36</sub>A was found to have a MIC for penicillin G of .015 µg/ml, the autolysin-defective mutant of 0.2 µg/ml, the Wicky strain of *S. sanguis*, of 0.13 µg/ml, and the *S. aureus* 502A, of .075 µg/ml broth.

### *Leukocyte Preparation*

PMNs were separated from the heparinized (10 U/ml) peripheral blood of healthy consenting volunteers, from a patient with CGD [9], and a patient with leukocyte MPO deficiency [10]. The blood was processed by centrifugation on Ficoll-Hypaque and dextran sedimentation, with hypotonic lysis of the erythrocytes as previously described [11]. The leukocyte-rich supernatants were centrifuged at 150 g for seven minutes at 4°C, the cells enumerated by hemocytometer and resuspended in antibiotic-free HBSS to a final concentration of 5 × 10<sup>6</sup>/ml. PMNs comprised 80 to 95 percent of the final cell population and constituted 98 percent of the phagocytic cells.

### *Preparation of Bacteria*

Samples were taken from each stock culture, diluted 1:100, and incubated in fresh TSB at 37°C for two hours (three hours for *S. sanguis*) to place the bacteria in the logarithmic growth phase. At this point, each bacterial suspension was divided into two equal aliquots, and penicillin G, at a final concentration of 1/4 to 1/32 of the MIC of the respective bacteria, was added to one and sterile TSB to the other (control). Both bacterial suspensions were allowed to grow for another two hours (three hours for *S. sanguis*). At the end of that period, the bacteria were removed from the antibiotic-containing or control broth by centrifugation at 2,200 g for ten minutes, washed by suspension in MHS, recentrifuged, and finally resuspended in HBSS. They were adjusted to 2.5 × 10<sup>8</sup>/ml viable counts after gentle sonication (20,000 Hz output of 35 W for ten seconds, Brown Sonic Power Co., Danbury, CT). This amount of sonication was sufficient to disrupt large clumps of staphylococci and to break the streptococcal chains into diplococci or short chains of four to six organisms, which were equivalent for untreated and penicillin-treated organisms. The bacterial inoculum was standardized visually by turbidity. When desired, <sup>14</sup>C labeling of bacterial proteins was

achieved by performing the four-hour (or six-hour for *S. sanguis*) incubation in the presence of 2–3  $\mu\text{Ci}$  (3,000,000 CPM) of  $^{14}\text{C}$ -labeled mixed L-amino acids [1].

### *Bactericidal Assays*

1 ml suspensions containing  $2.5 \times 10^6$  PMN and approximately  $2.5 \times 10^7$  bacteria were incubated in HBSS at  $37^\circ\text{C}$  in the presence of 1 percent albumin and 10 percent serum as a source of opsonins. When cytochalasin B was used, the cells were preincubated with 5  $\mu\text{g}/\text{ml}$  of the compound for ten minutes at  $37^\circ\text{C}$  before the addition of the bacteria. The bacterial inoculum was equalized for treated and untreated organisms as determined by Petroff-Hauser chamber counting.

Initial samples were taken immediately after addition of the bacteria to the tubes, which were then capped and rotated at  $37^\circ\text{C}$ . Killing of the inoculum was measured at various times by a previously described method [1,11]. The percentage of survival was calculated by dividing the number of colony-forming units at each time by the number at 0 minutes and then multiplying by 100. From this figure, the percentage of killing could be calculated by subtraction from 100. Appropriate cell-free controls were run for each assay.

### *Phagocytosis Assays*

The uptake (binding plus phagocytosis) of viable  $^{14}\text{C}$ -labeled bacteria by the cells was assessed as previously described [1,11]. Leukocytes in a final concentration of  $2.5 \times 10^6$  ml were placed in  $12 \times 75$  mm glass borosilicate tubes with HBSS, 1 percent albumin, and 10 percent serum for opsonization. After preincubation for ten minutes at  $37^\circ\text{C}$ , the bacterial suspensions ( $2.5 \times 10^7$ ) were added to the mixture at 20-second intervals. The capped tubes were allowed to rotate for 30 minutes, at which time phagocytosis was stopped by flooding the suspensions with 2 ml of ice-cold HBSS containing 10 percent fetal calf serum and 0.2 M NaF and immediately placing them on ice. Preparation of the cell suspension for radioactive counting was as previously described [1]. The percentage of organisms associated with the cells could be calculated by dividing cell-associated counts per minute by the counts per minute in the original inoculum and multiplying by 100. Similarly, the number of bacteria associated with the cells could be obtained from calculations of the specific activity of the radiolabeled organisms.

On multiple occasions, the bactericidal and phagocytosis assays were run simultaneously with the same inoculum. In experimental and control suspensions, the fraction of bacteria associated with the cells that were killed could thus be calculated. All the experiments were run in duplicate as well as with simultaneous cell-free controls.

### *Statistical Analysis*

The student *t*-test for paired samples was used for analysis. Values of  $p < 0.05$  for differences between control and experimental preparations were considered statistically significant.

## RESULTS

### *Growth and Amino Acid Labeling of Pneumococci and S. sanguis*

Organisms incubated in the presence of  $1/4$  to  $1/32$  the MIC of penicillin G multiplied at the same rate as controls; for example, for wild-type pneumococcus, the bacterial

TABLE 1  
Effect of SubMIC Penicillin (PCN) Pre-Treatment on Uptake and Killing of *S. pneumoniae* R<sub>36</sub>A by Normal and Cytochalasin B-Treated PMNs<sup>a</sup>

	Untreated Bacteria	¼ MIC PCN-Treated Bacteria
	Normal Cells	
% uptake (7)	60.6 ± 3.78	60.3 ± 4.44
% killed (8)	78.5 ± 5.56	79.9 ± 5.08
	Cyto B-Treated Cells	
% uptake (9)	43.41 ± 3.29	43.18 ± 2.84
% killed (16)	22.72 ± 4.67	45.6 ± 4.51 <sup>b</sup>

<sup>a</sup>Data are expressed as the mean ± SEM of the number of experiments shown in parentheses. The incubation period was 30 minutes. The inoculum measured an average of  $2.08 \times 10^7 \pm 0.18$  vs.  $2.05 \times 10^7 \pm 2.2$  bacteria for untreated vs. treated organisms, respectively.

<sup>b</sup> $p < 0.01$  student's *t*-test for paired samples in comparison with simultaneously run untreated organisms

population reached at two hours averaged  $1.89 \times 10^7$  for the bacteria incubated in antibiotic-free media and  $2.03 \times 10^7$  for the organisms exposed to ¼ MIC concentration of the penicillin ( $n = 16$ ). The subsequent viability of both control and treated organisms in suspensions incubated without PMNs was equivalent as long as 1 percent albumin was present in the medium. Similar findings were noted for the autolysin-defective mutant and the *S. sanguis* strain. In the experiments using <sup>14</sup>C amino acid labeling of bacteria, wild-type pneumococci treated with ¼ the MIC of penicillin G accumulated a slightly higher mean number of counts per minute per  $10^6$  bacteria (32.06) than untreated bacteria (26.14;  $n = 12$ ;  $p > .2$ ). This difference was less than our previous findings with *S. aureus* 502A [1].

While the *S. sanguis* strain did not replicate as quickly as the pneumococci, incubation in subMIC antibiotic concentrations of penicillin, up to ¼ MIC, did not affect growth or labeling with <sup>14</sup>C amino acids. Similar findings were noted for the autolysin-defective pneumococcal mutant.

#### *Killing of Pneumococcus (R<sub>36</sub>A) by Normal and Cytochalasin B-Treated PMNs*

Both normal and cytochalasin B-treated PMNs were used in the bactericidal-phagocytosis assays. Since viability of pneumococci was found to be poor in HBSS with 10 percent pooled human serum, 1 percent albumin was added to all bacterial-cell suspensions. These conditions did not alter phagocytic or bactericidal ability of the PMNs. Furthermore, survival of treated and untreated organisms in this medium was equivalent.

As with *S. aureus* [1], most bacterial uptake and killing occurred in the first 30 minutes; hence, since incubation for longer time periods did not alter the differences [1] all data presented are for a 30-minute time period. Pretreatment for two hours with ¼ MIC of penicillin had no significant effect on phagocytosis or killing of the rough pneumococcus by normal PMNs. Essentially all organisms which became cell-associated were killed with or without penicillin pretreatment (Table 1); in fact, the percentage killed as measured by pour plate technique was higher than that taken up as calculated by cell-associated <sup>14</sup>C amino acid counts. In contrast, treatment of the cells with cytochalasin B significantly (and equally) reduced the uptake of both untreated and penicillin-pretreated bacteria ( $p < .05$ ; *t*-test). Relative to phagocytosis, killing was impaired for only the untreated bacteria, however ( $p < .01$  for paired

TABLE 2  
Effect of subMIC Penicillin (PCN) Pretreatment on Uptake and Killing of Lysin-Defective Streptococci by Cytochalasin B-Treated PMNs<sup>a</sup>

	Untreated Bacteria	1/4 MIC PCN-Treated Bacteria
	<i>S. pneumoniae</i> R <sub>36</sub> A Lysin-Defective Strain	
% uptake (5)	65.9 ± 4.9	66.4 ± 5.4
% killed (5)	23.4 ± 3.5	40.8 ± 3.3 <sup>b</sup>
	<i>S. sanguis</i> , "Wicky" Strain	
% uptake (3)	63.4 ± 10.6	66.6 ± 10.3
% killed (3)	24.2 ± 3.1	51.0 ± 11.6 <sup>b</sup>

<sup>a</sup>Data are expressed as the mean ± SEM of the number of experiments shown in parentheses. The inoculum was equivalent for treated or untreated organisms. The incubation period was 30 minutes.

<sup>b</sup>*p* < 0.01 student's *t*-test for paired samples in comparison with simultaneously run untreated organisms

samples). In the presence of cytochalasin B, as with normal PMNs, all treated pneumococci taken up by the cells were killed vs. only a fraction (52 percent) of the untreated organisms.

In experiments not shown, pretreatment with as little as 1/8 of the MIC resulted in augmented killing by cytochalasin B-treated PMNs. The antibiotic effect was lost at 1/16 of the MIC or less. The effect was also abolished when penicillinase was added to the penicillin-containing medium during the pretreatment period.

#### *Phagocytosis and Killing of Lysin-Defective Pneumococcus and S. sanguis, Strain Wicky*

Similar to the wild-type pneumococcus R<sub>36</sub>A, no enhancement of killing of the antibiotic-treated lysin-defective pneumococcus or *S. sanguis* was observed when normal PMNs were used, since essentially all bacteria taken up by the cells were killed regardless of pretreatment. Treatment of the cells with cytochalasin B inhibited bacterial uptake equally for treated and non-penicillin treated organisms, and thus cytochalasin B-treated cells were used for subsequent comparative studies. Table 2 compares the cellular uptake and killing of the autolysin-defective pneumococcal strain and the Wicky strain of *Streptococcus sanguis*. In these experiments, the bacterial inoculum of the lysin-defective pneumococcus was lower than that of the wild-type strain, which accounts in part for the greater percentage of uptake than that found for wild-type organisms and shown in Table 1. Penicillin pretreatment approximately doubled killing of both of the autolysin-defective strains; the percentage of organisms killed was less than that taken up, however, in contrast to the wild-type R<sub>36</sub>A pneumococcus and our previous findings with *S. aureus* 502A [1].

#### *Role of PMN Oxidative Pathways in Killing Penicillin-Treated Gram-Positive Cocci*

To assess the role of various oxidative pathways in the killing of untreated and penicillin-pretreated gram-positive cocci, studies employing sodium azide or exposure to MPO-deficient or CGD PMNs were carried out.

The addition of sodium azide (1 mM concentration) to the incubation mixtures to block MPO activity did not significantly alter the uptake of any of the bacterial strains by cytochalasin B-treated PMNs (data not shown). In contrast, the effects of azide on bacterial killing were strain-dependent (Table 3). For example, azide reduced killing of untreated pneumococci by a mean of 34.4 percent and that of the penicillin-

TABLE 3  
Role of Oxidative Mechanisms in Killing of Untreated and subMIC Penicillin-Treated Bacteria by Cytochalasin B-Treated PMNs<sup>a</sup>

PMNs	Bacteria PCN Treatment	Organisms % Killed		
		<i>S. aureus</i>	<i>S. pneumoniae</i> R <sub>36</sub> A	<i>S. sanguis</i>
Control	—	28.7 ± 1.9 (7)	25.6 ± 2.8 (4)	25.8 ± 2.2 (5)
	+	57.7 ± 4.0 (7) <sup>b</sup>	45.7 ± 8.1 (4) <sup>b</sup>	66.6 ± 6.9 (5) <sup>b</sup>
Azide-Treated	—	26.0 ± 2.2 (4)	16.8 ± 2.7 (4)	15.2 ± 3.6 (5)
	+	46.6 ± 7.4 (4) <sup>b</sup>	20.2 ± 7.5 (4)	33.7 ± 11.3 (5)
MPO-Deficient	—	14.0 (1)	19.6 (1)	—
	+	53.0 (1)	14.3	—
CGD	—	18.0 ± 0.05 (2)	21.7 (1)	—
	+	31.7 ± 3.3 (2) <sup>b</sup>	24.6	—

<sup>a</sup>Data are expressed as the mean ± SEM of the number of experiments shown in parentheses for multiple experiments. Single values are shown for single experiments. The incubation period was 30 minutes.

<sup>b</sup>*p* < .05 paired *t*-test when compared to non-penicillin-treated controls

pretreated organisms by 55.7 percent. The net effect was to abolish the differences in susceptibility to killing of these organisms created by exposure to the antibiotic. Incubation of PMNs with azide reduced killing of untreated *S. sanguis* by a mean of 41 percent vs. 44.0 percent for penicillin-pretreated organisms. The net effect was to retain the increase in susceptibility to killing of antibiotic-exposed organisms, although the absolute differences were reduced. When killing of *S. aureus* was studied with azide-treated cells, a modest decrease in killing of penicillin-treated organisms was observed, but the penicillin effect was retained.

Studies with cells from a patient with MPO deficiency and a second patient with CGD confirmed the differences between mechanisms used to kill penicillin-pretreated pneumococci and staphylococci. The enhanced killing of penicillin-pretreated pneumococci was abolished when either MPO-deficient or CGD PMNs were employed. While CGD- or MPO-deficient PMNs killed staphylococci less effectively than normal cells, the differences in susceptibility to killing between penicillin-pretreated and untreated organisms were retained.

The potential role of oxidative mechanisms in the killing of *S. aureus* was further explored, using several oxygen reduction product scavengers in incubations with cytochalasin B-treated cells. Augmented killing of penicillin-pretreated bacteria was preserved in the presence of scavengers of O<sub>2</sub><sup>-</sup> (SOD), H<sub>2</sub>O<sub>2</sub> (catalase), OH<sup>·</sup> and <sup>1</sup>O<sub>2</sub> (catalase and SOD) (Table 4).

## DISCUSSION

We have developed a model to study the effect of subMIC concentrations of antibiotics on neutrophil killing of gram-positive cocci. The model is based on the hypothesis that modification of particular bacterial target sites by antibiotics may increase susceptibility of these organisms to phagocytosis or killing by PMNs. We have shown that antibiotics which modify the cell wall of staphylococci rather than those with an intracellular site of action are more likely to increase susceptibility to killing by PMNs [1,2]. Presumably, the action of cell-wall active antibiotics creates a lesion in the organism at a site which is readily accessible to the microbicidal products of the cells. The use of doses of antibiotics which are below the minimal concentration

TABLE 4  
Effect of Oxygen Product Scavengers on Killing of *S. aureus* 502A by Cytochalasin B-Treated PMNs<sup>a</sup>

Additions	Bacteria	
	Untreated	¼ MIC PCN-Treated <sup>b</sup>
None (14)	29.3 ± 4.9	62.8 ± 2.8
SOD (50 µg/ml) (3)	27.5 ± 0.2	65.3 ± 4.6
Catalase (4,000 µ/ml) (2)	26.5 ± 12.5	70.8 ± 3.6
Catalase + SOD (5)	33.1 ± 7.6	56.3 ± 10.0

<sup>a</sup>Data expressed as mean ± SEM of percentage killed at 30 minutes. Numbers in parentheses represent the number of experiments.

<sup>b</sup>All values significantly greater than with untreated bacteria ( $p < .05$ , *t*-test)

required to inhibit growth has permitted a clearer distinction between antibiotic and PMN effects. Modification of the surface of *S. aureus* by pretreatment with cell-wall active antibiotics increases their susceptibility to killing by PMNs to a point that killing occurs with inhibition of phagosome formation and without modification of bacterial uptake or phagocytosis by normal or cytochalasin B-treated cells [1].

*S. aureus* are catalase-positive organisms which contain autolytic enzymes that could be involved in the combined effect of antibiotic and neutrophil microbicidal mechanisms. Furthermore, the presence of catalase could have modified the susceptibility of these organisms to oxidative killing by PMNs [12]. To assess the role of catalase and to determine a role for the autolysins in this model, we used catalase-negative organisms, for which an autolysin-defective strain was available, and exposed them to penicillin and PMNs in the same fashion as with *S. aureus*. Treatment of streptococci with penicillin causes secretion of cell-wall polymers into the surrounding medium, including capsular polysaccharide [13,14]. These findings have been observed with bold wild-type and lysin-defective pneumococcus [4,13,14]. The use of rough strains of these organisms reduced the possibility that changes in killing by PMNs following penicillin pretreatment could be ascribed to loss of capsular polysaccharide and alterations in phagocytosis [15]. Since both bacterial uptake and killing by PMNs were measured simultaneously in the same preparation in most experiments, a comparison could be made between both parameters and the fraction taken up-killed could be calculated.

Pretreatment of the streptococcal strains with penicillin G at various fractions of the MIC caused no appreciable change in their replication or incorporation of mixed <sup>14</sup>C-amino acids. Normal PMNs ingested and killed both treated and untreated bacteria avidly, and no effect of penicillin pretreatment could be observed. This contrasted with our earlier findings with *S. aureus* 502A in which the native resistance of these organisms to intracellular killing by normal cells was overcome by subMIC penicillin pretreatment. The increase in the percentage of streptococcal organisms killed above those measured to be taken up by the cells (refer to Table 1) could represent extracellular killing as reported by Weiss et al. [16] or a failure to separate cleanly intracellular aggregates of viable organisms to be counted as separate colonies.

Cytochalasin B treatment of PMNs inhibits phagosome formation, but not the ability of organisms to adhere to phagocytic receptors [1,17] and to trigger a



respiratory burst [18,19] and lysosomal degranulation [19]. It offers the opportunity to segregate events which occur following binding of various stimuli to the cell surface from those which are dependent upon phagosome formation. Consistent with previous observations [1,17,20], when cells treated with cytochalasin B were used, cellular uptake was moderately and killing of untreated bacteria markedly impaired. Cytochalasin B-treated PMNs took up opsonized untreated and penicillin-pretreated streptococci equally well, a finding we previously noted for staphylococci [1]. A significant difference in killing could be demonstrated between penicillin-treated and untreated bacteria, however, so that essentially all cell-associated penicillin-treated organisms were killed vs. approximately one-half of the untreated cocci. These effects of penicillin pretreatment were observed in both autolysin-replete and -deficient strains, thereby excluding an important cooperative role for bacterial autolysins as mediators of the effect with the PMN microbicidal mechanisms. Furthermore, the equivalent cellular uptake of untreated and treated bacteria distinguishes these effects of penicillin pretreatment from those reported for group A or B streptococci [15,21].

The neutrophil microbicidal mechanisms responsible for effecting the enhanced killing of the pretreated organisms were explored, using cytochalasin B-treated cells, concentrating in particular on the potential role for oxidative pathways. Sodium azide was employed to inhibit the MPO activity of normal cells [22,23]. Furthermore, cells from patients totally deficient in MPO activity or the ability to generate oxygen-reduction products during phagocytosis (CGD) were also used. The results indicated that oxidative pathways were important in mediating the enhanced killing of the catalase-negative pneumococci since the effect was abrogated by azide and lost in both MPO-deficient and CGD PMNs. SubMIC penicillin treatment thus rendered these organisms specifically more susceptible to killing by the MPO-H<sub>2</sub>O<sub>2</sub> microbicidal system of the neutrophil. The enhancement was such that this system could operate effectively against organisms bound to cells and not enclosed in phagosomes, the consequence of cytochalasin B treatment [1,17,18].

The precise nature of the target site modification created by penicillin pretreatment which promoted this expression of the MPO-H<sub>2</sub>O<sub>2</sub> system against pneumococci remains to be elucidated. The possibilities range from better access of MPO or H<sub>2</sub>O<sub>2</sub> to this target site, improved presentation of amino groups for chlorination reactions [24,25] or iron centers for oxidative attack by hypochlorous acid [26], or some other yet-to-be-defined mechanism.

Whatever the nature of this mechanism, it appears to operate independently of microbial catalase, which is absent in pneumococci. Evidence for this is provided by the observation that the related catalase-negative streptococcal species, *S. sanguis*, and *S. aureus*, catalase-positive, both retained enhanced susceptibility to killing after penicillin pretreatment in the absence of cellular MPO activity. Specifically, while killing was reduced slightly by inhibition of MPO with azide or with MPO-deficient or CGD cells, the penicillin enhancement was preserved. Furthermore, the addition of scavengers of superoxide dismutase (SOD) and H<sub>2</sub>O<sub>2</sub>, to the medium did not modify the ability of cytochalasin B-treated normal cells to kill the staphylococci. Enhanced killing following penicillin pretreatment was also found in the presence of both SOD and catalase, a condition in which both <sup>1</sup>O<sub>2</sub> and <sup>•</sup>OH formation by the Haber-Weiss reaction would be impaired [27].

These findings imply that penicillin pretreatment of *S. sanguis* and *S. aureus* augments the action of non-oxidative PMN microbicidal mechanisms against these

organisms [28]. The nature of these mechanisms and their target(s) requires clarification. In preliminary studies, the survival of subMIC penicillin pretreated staphylococci at an acid pH was equivalent to untreated bacteria. A mildly increased susceptibility to the action of lysozyme has been observed similar to findings reported in the past by Warren and Gray [29]. A cooperative role for lytic serum complement factors has been excluded [30]. The potential action of antibacterial cationic proteins, perhaps acting in cooperation with lysozyme, requires further evaluation [31].

Whatever the precise mechanisms involved, these studies demonstrate that antimicrobial effects against bacteria may profoundly alter their interaction with essential host defense mechanisms beyond an ability to kill organisms or impair their growth. This seems to be particularly true of compounds which modify bacterial surface properties. The clinical implications of these findings justify further exploration of the responsible mechanisms and an expansion of these approaches to other organisms and other antimicrobial classes.

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