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O Antigen and Lipid A Phosphoryl Groups in Resistance of *Salmonella typhimurium* LT-2 to Nonoxidative Killing in Human Polymorphonuclear Neutrophils

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We have compared the intraleukocytic survival of isogenic strains of *Salmonella typhimurium*, whose outer membrane lipopolysaccharide differed in O antigen and lipid A composition and whose susceptibility to nonoxidative antimicrobial granule proteins of human polymorphonuclear neutrophilis (PMN) could be established. We found that the order of resistance to the bactericidal activity of intact PMN of the three bacterial strains utilized closely resembled their ordered resistance to the purified human cationic antimicrobial 57,000-dalton protein (CAP57). LT-2, a smooth wild-type strain, was far more resistant than SH9178, its rough (Rb LPS) mutant. It was most significant that SH7426, a polymyxin B-resistant *pmrA* mutant of SH9178, not only was substantially more resistant to CAP57 and to intraphagocytic killing than SH9178 but also came close to being as resistant as LT-2. These experiments confirm earlier work that showed the importance of the glycosyl groups of O antigens of *S. typhimurium* for their resistance to O_2-independent antimicrobial phagocytosis by PMN. The surprising result was that a rough strain, very susceptible to bactericide, became substantially more resistant when a mutation led to its lipid A phosphoryl groups being 100% substituted with amino pentoses. Yet unresolved is whether the protection is due to the loss of negative charges on the lipid A, the substitution of sugar molecules in vulnerable loci in the outer membrane, or both.

Previous investigations have clearly illustrated the importance of lipopolysaccharide (LPS) in the resistance of bacteria to the bactericidal activity of polymorphonuclear neutrophils (PMN) and their fractionated granule proteins. Rest et al. (7) showed that smooth strains of *Salmonella typhimurium* were much more resistant to killing by lysosomal fractions of neutrophils. Subsequent work by Modrzakowski and Spitznagel (5) showed that purified LPS from smooth *S. typhimurium* bound several cationic proteins and inhibited their bactericidal activity. With a whole cell phagocytosis and killing assay, Okamura and Spitznagel (6) showed that smooth *Salmonella* strains were more resistant to killing than were rough strains in an anaerobic environment.

We have recently discovered that mutations in the *S. typhimurium* chromosome that modulate the negative charges on phosphoryl groups of lipid A appear to enhance their capacity to survive in the presence of neutrophil granule proteins such as cationic antimicrobial proteins CAP37 and CAP57 acting in vitro (9, 12). We now report studies on the effects of the same mutation on the resistance of *S. typhimurium* to microbicidal granule proteins acting intracellularly. We present detailed comparisons of the capacity of the mutants and their wild-type parents to survive when introduced together in a mixed suspension to both untreated and sphingosine-treated (nonoxidizing) PMN monolayers.

Resistance of *S. typhimurium* to cationic, amphipathic antimicrobial proteins is complex. Shafer and colleagues (9) have suggested that the hydrophilic side chain of smooth LPS presents a thermodynamic barrier to amphipathic, antimicrobial proteins and to polymyxin B. They suggested that one reason rough strains are less resistant to such antimicrobial proteins is the absence of this barrier, which reveals more hydrophobic domains of the outer membrane. Shafer et al. (9) and Farley et al. (3) have more recently postulated that the cationic proteins of PMN form salt bonds with the unsubstituted phosphoryl groups of lipid A in the bacterial outer membrane. That suggestion was based on studies of the resistance of an isogeneic pair of semirough *S. typhimurium* mutants (Rb) (*pmrA*$^+$ and *pmrA*) that differ in the extent of arabinosylation of the phosphoryl groups of their lipid A. The *pmrA* mutants were known to be resistant to the cationic, amphipathic antibiotic polymyxin B (11) and were shown by Shafer et al. (9) and Farley et al. (3) to be similarly resitant to the cationic granule proteins of PMN. Now, to determine resistance to killing conferred by the *pmrA* mutation in normal PMN which possess their full bactericidal armament or are restricted to killing because of O_2-independent mechanisms, we have studied the killing of wild-type *S. typhimurium* LT-2, its *pmrA*$^+$ semirough mutant SH9178, and an isogeneic derivative of SH9178, *pmrA* SH7426, in normal or sphingosine-treated (O_2-independent killing) PMN (P. Stinavage and J. K. Spitznagel, J. Immunol. Methods, in press).

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MATERIALS AND METHODS

Bacteria. Table 1 summarizes the bacterial phenotypes and sources of the bacteria used in this study.

Sphingosine. D-sphingosine from bovine brain cerebrosides was obtained from the Sigma Chemical Co., St. Louis, Mo. Sphingosine stock solution (5 mM) was prepared as an equimolar solution with fatty acid-free bovine serum albumin as follows. A 50 mM solution of sphingosine was prepared in absolute ethanol. One milliliter of this solution was then added to 9 ml of a 5 mM solution of fatty acid-free bovine serum albumin. Sphingosine was added to PMN suspensions to a final concentration of 20 μM prior to the

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TABLE 1. *Salmonella* strains employed in this study

S. typhimurium strain (mutant)	LPS chemotype	Source
LT2 (*pmrA*$^+$)	Smooth	Salmonella Genetic Stock Center, Calgary, Alberta, Canada
SH9178 (*pmrA*$^+$)	Rb, *rfaJ* PB sensitive	H. Makela
SH7426 (*pmrA*)	Rb, *rfaJ* PB resistant	H. Makela

45-min attachment period. Sphingosine was also added to bacterial suspensions to a final concentration of 20 μM prior to the addition of the bacterial suspensions to the PMN monolayers.

Serum. We obtained fresh venous blood from normal volunteers after obtaining their informed consent. The blood was allowed to clot and then was centrifuged at 4,700 × *g* for 10 min. Serum was drawn off and frozen at −70°C in 1-ml portions.

Radiolabeling. [^{3}H]uracil (Lot #268599) and [^{35}S]methionine-cysteine (Tran ^{35}S label, Lot #152:140-B) were obtained from ICN Radiochemicals, Irvine, Calif. [^{14}C]glucose (Lot #279761) was obtained from Sigma Chemical Co. [^{3}H]uracil labeling was accomplished as follows: glucose from a 40% stock solution was added to 10 ml of no carbon E medium (2) to obtain a final glucose concentration of 0.1% unlabeled glucose. [^{3}H]uracil was then added to a final activity of 1 μCi/ml. This was inoculated with a single colony of the bacteria picked from an overnight culture on LB agar (2). The broth was then incubated overnight at 37°C.

[^{35}S]methionine labeling was carried out as follows. Methionine-free assay media (catalog no. 0423-15) was obtained from Difco Laboratories, Detroit, Mich. Radiolabeled methionine (Tran ^{35}S label; ICN) was added to 10 ml of this medium to obtain an activity of 1 μCi/ml. Unlabeled methionine was added to a final concentration of 0.2 mM. Again, this broth was inoculated with a single colony picked from an overnight LB agar plate and incubated at 37°C overnight.

After overnight incubation, the bacterial cultures were centrifuged at 4,700 × *g* for 15 min and the supernatant was discarded and suspended in cold phosphate-buffered saline. This wash procedure was repeated three times to remove extracellular radioactivity, and the final suspension was in Hanks buffered salt solution (HBSS). An optical density at 650 nm was determined on each culture and adjusted to be equal in each culture. Equal volumes of the two cultures were then mixed. This suspension was then opsonized with normal human serum. Portions of these suspensions were then removed for subsequent determination of radiolabeling efficacy and bacterial viability. Two hundred microliters of this suspension was added to PMN monolayers to determine phagocytosis and killing.

In order to differentiate radiolabel counts in each experiment, one strain was labeled with [^{3}H]uracil and the other was labeled with either [^{14}C]glucose or [^{35}S]methionine. In at least one of the trials done for each experiment presented (see Fig. 2 through 5), the organisms were labeled with the alternate compound to ensure that the choice of precursor did not affect the conclusions.

Opsonization. Thawed normal human serum was added to the bacterial suspensions to a concentration of 8%. Suspensions were then incubated at 2°C for 60 min.

Purification of PMN. We obtained fresh venous blood from normal volunteers after obtaining their informed consent. The PMN were separated on a Ficoll density gradient (Lymphocyte Separation Medium; Litton Bionetics, Kensington, Md.). The pellet containing erythrocytes and PMN was dextran sedimented. Dextran supernatant was centrifuged, and remaining erythrocytes were removed by hypotonic lysis. This preparation was 98% PMN by Wright stain smear. PMN numbers were determined by Coulter count and adjusted to 5 × 10^6 cells per ml. Two hundred microliters of this suspension was added to the experimental wells in Corning 25860 microdilution 96-well flat-bottomed plates and allowed to adhere for 45 min.

Viability determinations. Appropriate serial dilutions were prepared from the opsonized bacterial suspensions and every experimental and control well. A 10-μl drop of each dilution was plated on LB plates. Colony counts were made after 18 to 24 h of incubation at 37°C.

Assay. The supernatant was removed from PMN monolayers, and the monolayers were washed once with cold HBSS. (Sphingosine-treated cells were washed with HBSS containing 20 μM sphingosine). Supernatant and wash solutions were reserved and kept at 2°C for later cell count determinations. Two hundred microliters of the bacterial suspensions was then added to triplicate experimental and duplicate (no PMN) control wells. Duplicate radiolabel and duplicate viability control wells at each time point were included in the protocol. Samples were removed from radiolabel control wells to determine the number of radioactive counts that nonspecifically adhered to the wells, and samples from viability controls were used to determine the extent of bactericidal activity by the opsonic agent. Plates were then centrifuged at 275 × *g* for 10 min to ensure maximal bacteria-to-PMN contact. Plates were then incubated with the bacterial suspension for the 0-, 15-, and 30-min time points.

One hundred microliters of the supernatant was removed from the experimental and control wells and added to 3 ml of Beckman HP-b scintillation cocktail (Beckman Instruments, Fullerton, Calif.). Scintillation counting occurred at the 0-, 15-, and 30-min time points to determine the bacterial numbers remaining in suspension that had not contacted the monolayers. The remaining supernatant was removed, and monolayers were washed three times with cold HBSS to remove extracellular bacteria. Monolayers were then disrupted by the addition of 200 μl of 0.5% Triton X-100. Triton X-100 at this concentration has previously been shown not to affect bacterial viability (6). Rapid pipetting with a micropipettor ensured that the monolayer was disrupted and that the remaining bacteria were uniformly suspended. One hundred microliters of this suspension was added to 3 ml of scintillation cocktail for determination of cell-associated bacteria. Another portion of 20 μl of the remaining suspension was added to 1,980 μl of sterile distilled water for serial dilution and subsequent bacterial viability determinations.

After 30 min of incubation, the bacterial suspension was removed from plates that had been incubated for 60 min and wells were washed three times with HBSS to remove extracellular bacteria. After the final wash, 200 μl of HBSS was again added to the wells and plates were reincubated for the remainder of the experimental period. This was done in an effort to control the replication of uningested organisms that were still present in the supernatant at this point. At the end of the experimental period, the supernatant was removed and the monolayers were washed once with HBSS and disrupted in exactly the same manner as previously described. Portions for scintillation counting and viability determinations were also removed as described previously.

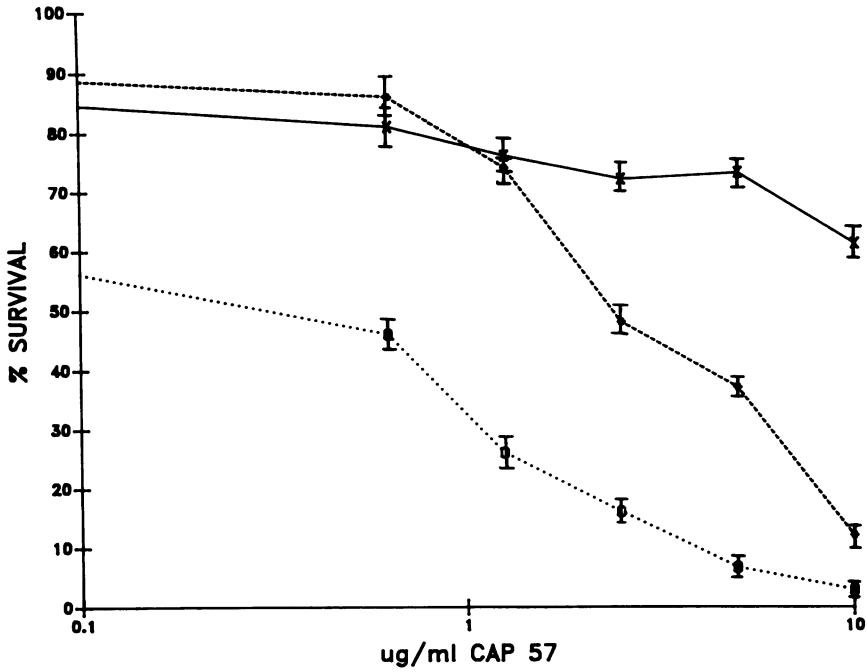

FIG. 1. Comparison of survival of *S. typhimurium* strains incubated with the purified cationic neutrophil granule protein, CAP57, at pH 5.5 for 60 min. Symbols: X——X, sensitivity of strain LT-2 (smooth LPS; 50% lethal dose, >10 μg/ml); 0·····0, sensitivity of strain SH9178 (Rb LPS; *pmrA*$^+$; 50% lethal dose, <0.625 μg/ml); *·····*, sensitivity of strain SH7426 (Rb LPS; *pmrA*; 50% lethal dose, ca. 2.5 μg/ml).

Determination of specific radioactivity of labeled organisms. Specific radioactivity was calculated from the viability determinations of the opsonized inocula by the following formula: radioactive counts/(number of viable organisms × 10^6) = radioactivity per 10^6 viable organisms.

Viability determinations. Appropriate serial dilutions were prepared from the opsonized bacterial suspensions and every experimental and control well. A 10-μl drop of each dilution was plated on tryptic soy agar. Colonial morphology was adequate to distinguish wild-type LT-2 from the semirough mutants and SH9178 from SH7426 and to distinguish the mutants from each other.

Preparation of 57,000-dalton cationic antimicrobial neutrophil granule protein and bactericidal assay. CAP57 was prepared from PMN granule extracts by the method of Shafer et al. (9) and as modified by Casey et al. (1). Antimicrobial assays were preformed by the method of Farley et al. (3).

Scintillation counting. Scintillation counts were determined by using library program #8 in a Beckman LS-8000 liquid scintillation counter. This program was designed to count dual-labeled ^{3}H- and ^{14}C- or ^{35}S-labeled samples by using H number (to measure counting efficiency) and automatic quench control.

Presentation of results and statistical analysis. Briefly, analysis of variance was performed on all bacterial viability data by using BMDP8V-Analysis of variance-Equal cell size mixed models (BMDP Statistical Software, Los Angeles, Calif.) by Michael Kutner, Department of Epidemiology and Biostatistics, Emory University. Points determined by analysis of variance were plotted, with bars representing standard errors of the mean.

RESULTS

Resistance of LT-2, SH9178, and SH7426 to purified CAP57. The killing of these three strains by purified 57,000-dalton cationic antimicrobial neutrophil granule protein was examined. Figure 1 illustrates the resistance of these strains to the purified protein at pH 5.5. SH7426 (*pmrA*) was better able to survive the action of CAP57 than was its parental strain SH9178 (*pmrA*$^+$). It is clear that SH9178 (*pmrA*$^+$) was the least resistant of the three to the actions of this protein. The smooth wild-type strain LT-2 strain was by far the most resistant to killing by CAP57. The concentrations of CAP57 resulting in a 50% loss of bacterial viability in 1 h for the three strains LT-2, SH7426, and SH9178 were >10, approximately 2.5, and <0.625 μg/ml, respectively. These results confirm the results found by previous investigations (3, 9, 10). The question that remained to be answered was whether LT-2, SH9178, and SH7426 would show similar resistance patterns to O_2-independent killing in intact PMN. We decided to make these comparisons with the bacteria labeled with radioisotopic tracers and with genetic phenotypic markers that enabled us to introduce them into the phagocytes in mixtures and to compare their capacities to survive side by side.

A potential confounding factor in side-by-side comparisons was the possibility that one strain might have an antagonistic or synergistic effect on the killing of the other strain in the same PMN. For example, since rough organisms are more hydrophobic than smooth organisms, this could play a role in enhancing the internalization of the rough, more hydrophobic organism and lead to their preferential uptake. Also, the effect of LPS on the internal environment of the PMN remains in question. To account for the possible effects of side-by-side comparison of rough versus smooth and rough versus rough, both rough organisms were first compared to the smooth wild type and then to each other.

Comparison of LT-2 (fully smooth wild type) with SH9178 (Rb, *pmrA*$^+$) in intact PMN. To determine if resistance to CAP57 would correlate with resistance to killing by intact

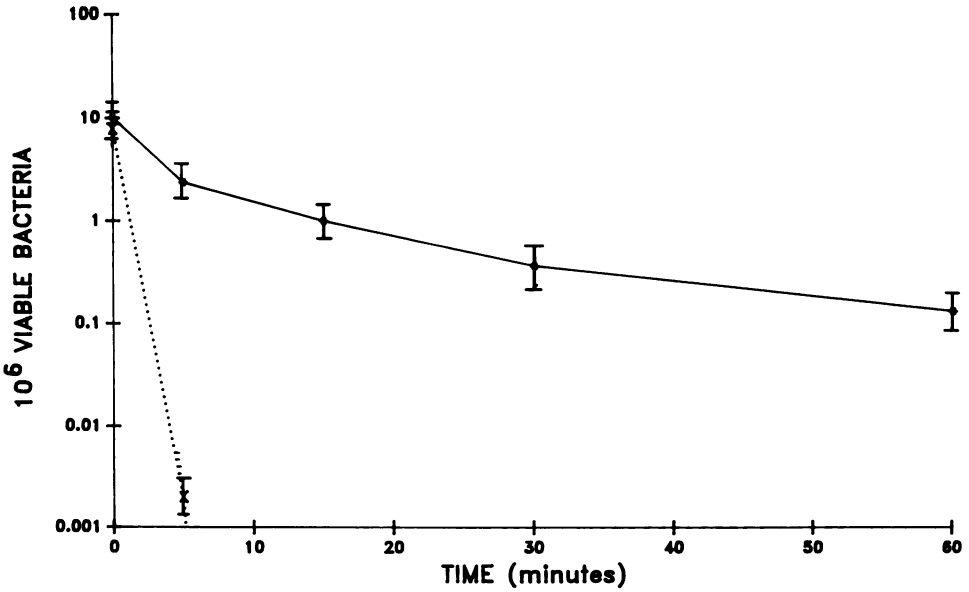

FIG. 2. Comparison of the survival of SH9178 (Rb, *pmrA*$^+$) and *S. typhimurium* LT-2 in the same set of PMN. Symbols: *——*, survival of LT2; X·····X, survival of SH9178. Points are represented as the mean plus or minus the standard deviation of triplicate determinations. The results of one representative experiment of three separate trials (on different days) are presented. The 0-min time point was determined by calculating the number of bacteria that had adhered to the neutrophil monolayer by using radiolabel counts.

PMN, we compared each of the semirough mutants to the wild type by using side-by-side phagocytosis, that is, phagocytosis in which mixtures of mutant and wild type were presented to a single PMN monolayer. Figure 2 shows the results of a bactericidal assay with intact PMN, comparing the wild-type LT2 (smooth LPS) with SH9178 (Rb, *pmrA*$^+$). The killing curves for these two organisms show that although the two strains were taken up in comparable numbers, the rough SH9178 was much more efficiently killed than was the smooth LT-2. The number of detectable viable SH9178 cells fell below the detection limits of the assay (10^3) by the 15-min time point and remained below the limits for the remainder of the 60-min assay period. These data showed, as expected, that the semirough SH9178 was far more sensitive to the actions of intact PMN than was its parent LT-2. The increased numbers of cell-associated bacteria shown here were due to a total bacteria-to-PMN ratio of approximately 40:1.

Comparison of LT-2 and SH7426 (Rb, *pmrA*) in intact PMN. To examine the effect of the *pmrA* mutation on the ability of *S. typhimurium* to survive within the phagolysosome, SH7426 was compared with LT-2 in side-by-side

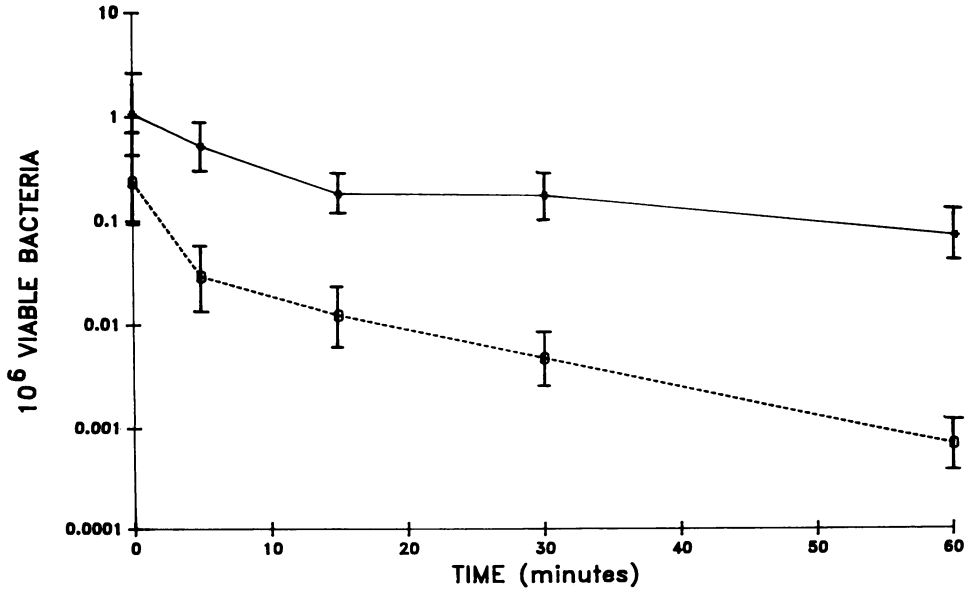

FIG. 3. Comparison of the survival of SH7426 (Rb, *pmrA*) and LT-2. Symbols: *——* survival of LT-2; 0——0, survival of SH7426. Points are presented as explained in the legend to Fig. 2. The results of one representative experiment of three separate trials (on different days) are presented. The killing kinetics of SH7426 more closely resemble those of the smooth LT-2 than did those of SH9178 in Fig. 2.

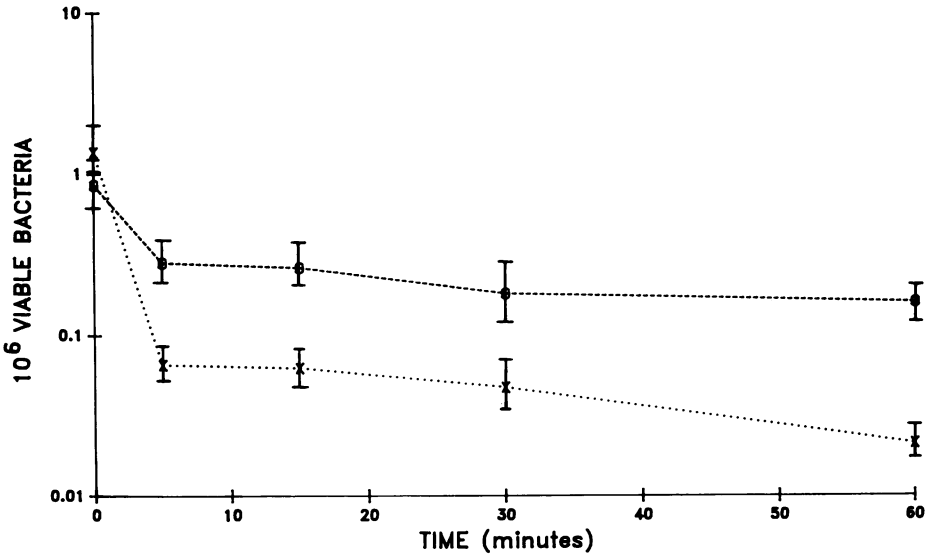

FIG. 4. Comparison of the survival of SH9178 (Rb, *pmrA*$^+$) and SH7426 (Rb, *pmrA*). Symbols: 0——0, survival of SH7426; X·····X, survival of SH9178. Points are presented as explained in the legend to Fig. 2. The results of one representative experiment of three separate trials (on different days) are presented.

phagocytosis. Figure 3 illustrates the results of the experiment. The bacteria-to-PMN ratio in these and subsequent experiments was approximately 10:1. The semirough *pmrA* mutant was more efficiently killed than was the smooth wild type, yet SH7426 clearly survived better than SH9178 (compare Fig. 2 and 3). Thus, it appeared that the *pmrA* mutation did enhance the ability of this semirough organism to survive, compared with its *pmrA*$^+$ parent.

Comparison of SH7426 (Rb, *pmrA*) to its parent SH9178 (Rb, *pmrA*$^+$). To determine if the *pmrA* mutation would enhance intraneutrophilic survival just as it enhanced resistance to CAP57, we compared SH7426 with its parent SH9178. This offered two advantages. First, the two strains are said to be isogenic except for *pmrA* (11); second, they are rough strains, and, as shown, rough strains are much less resistant to CAP57. Thus, use of rough strains allowed us to detect increases in resistance (if any) conferred by the mutations with greater sensitivity. Results are shown in Fig. 4. Clearly, SH7426 survived better in the bactericidal environment of intact neutrophils than did its parent SH9178.

Comparison of killing by sphingosine-treated PMN. To compare the killing of LT-2, SH9178, and SH7426 in PMN that were deprived of their ability to kill by oxidative mechanisms, we compared the killing of the two by sphingosine-treated PMN. We have previously verified that sphingosine inhibits the production of superoxide anion by PMN (P. Stinavage and J. K. Spitznagel, in press) and that sphingosine-treated neutrophils can be used in phagocytic tests as surrogates for neutrophils in an anaerobic chamber.

Figure 5 illustrates the results of the assays. They were very similar to those seen with normal, untreated PMN. Figure 5A shows the survival of LT-2 and SH9178 in sphingosine-treated PMN. LT-2 in these PMN underwent approximately a log drop in viable organisms, while viable SH9178 fell below the detection limits of the assay after the 30-min time point. Figure 5B compares the survival of LT-2 and SH7426 in sphingosine-treated PMN. In this case again, LT-2 showed approximately a log decrease in detectable viable numbers, while SH7426 underwent approximately a two-log decrease. While the numbers of viable SH7426 decreased dramatically over the assay period, numbers of SH7426 never fell below the detection limits of the assay. SH7426 and SH9178 are compared in Fig. 5C. These organisms show approximately the same resistance to killing by either sphingosine-treated or normal PMN.

Phagocytosis of radiolabeled organisms. Since we compared mixed suspensions of bacteria for the survival of mutants, it was necessary to compare cell association data to determine if the mutations affected the attachment of bacteria to the cells. These data are compared in Fig. 6. In every instance, more than 40% of the organisms introduced in the experiment were found associated with the monolayer after washing. No significant difference between the association of the three strains was observed in any of our experiments. The cell association of two different combinations of smooth and rough organisms (LT-2 and SH9178; LT-2 and SH7426) and a combination of two rough organisms (SH9178 and SH7426) all yielded cell associations that were similar. Organisms centrifuged onto the monolayers became firmly associated in greater numbers than they did at $1 \times g$. It is possible that the centrifuging also tended to abrogate differences in association, but we have no evidence for this. Furthermore, bactericide data support the interpretation that the bacteria had been internalized by the PMN. It is possible, although less likely, that the *pmrA* mutation allows adherence of the bacteria to the PMN but impairs phagocytosis, leading to an overestimation of viable *pmrA* organisms. Nonspecific binding of the bacteria to the microdilution wells was less than 10% (data not shown).

DISCUSSION

Rest et al. (7) showed that the resistance of *S. typhimurium* to neutrophil granule proteins was directly proportional to the length and complexity of the polysaccharides of outer membrane LPS. Later, Shafer et al. (9) and Farley et al. (3) showed that this principle directly applied to resistance to CAP37 and CAP57. Weiss et al. (12) obtained similar results for the BPI (bactericidal permeability increasing) factor. The 25 N-terminal amino acid residues of BPI factor are homol-

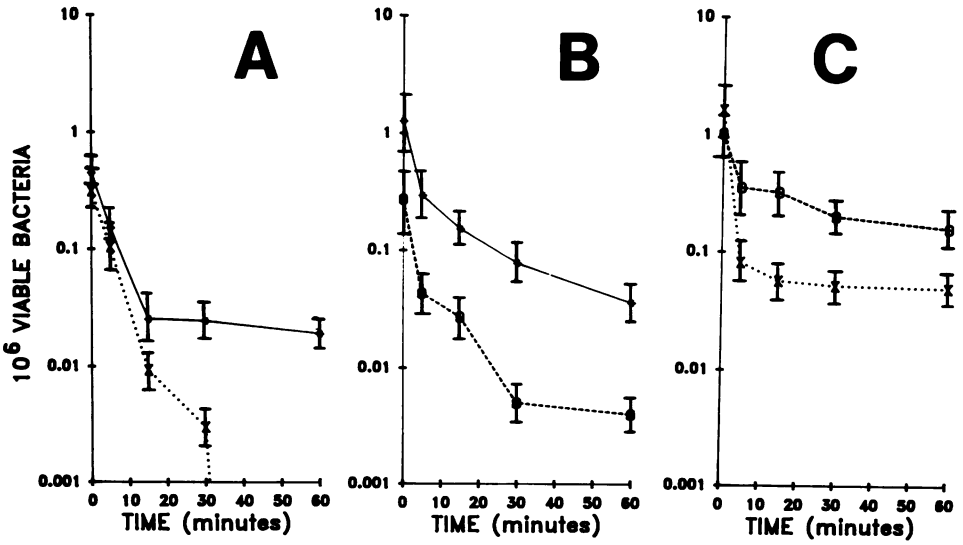

FIG. 5. Chart analysis of killing of SH9178, SH7426, and LT-2 in neutrophils treated with 20 μM sphingosine. (A) Comparison of the survival of SH9178 (Rb, *pmrA*$^+$) and *S. typhimurium* LT-2 in the same set of sphingosine-treated PMN. Symbols: *——*, survival of LT-2; X·····X, survival of SH9178. Points are presented as explained in the legend to Fig. 2. The results of one representative experiment of three separate trials (on different days) are presented. Viable numbers of SH9178 fell below the detection limits of the assay between the 30- and 60-min time points. (B) Comparison of the survival of SH7426 (Rb, *pmrA*) and LT-2. Symbols: *——*, survival of LT2; O——O, survival of SH7426. Points are presented as explained in the legend to Fig. 2. The results of one representative experiment of three separate trials (on different days) are presented. (C) Comparison of the survival of SH9178 (Rb, *pmrA*$^+$) and SH7426 (Rb, *pmrA*). Symbols: O——O survival of SH7426; X·····X, survival of SH9178. As seen here, the mutation in SH7426 increases survival of this organism in the sphingosine-treated PMN nearly as well as it does in untreated PMN.

ogous with those of CAP57, suggesting close similarlity in these proteins (J. K. Spitznagel, A. Pereira, and J. Pohl, unpublished results). Okamura and Spitznagel (6) showed with anaerobic neutrophils that as the carbohydrate content of bacterial lipopolysaccharide decreased, the bacteria became less resistant to the actions of oxygen-independent killing mechanisms.

More recently, Shafer et al. (9) found that *pmrA* (polymyxin B-resistant phenotype) is cross resistant to CAP57 and CAP37. (CAP37 has 57% homology with the N termini of certain serine proteinases but lacks homology with BPI or defensins; the 20 N-terminal amino acids of CAP37 are IVGGRKARPRQFPFLASIQN [A. Pereira, J. Pohl, and J. K. Spitznagel, submitted for publication]). Gabay et al. (4) have evidently confirmed the existence of CAP37, having described a similar protein under the name azurocidin. The change in the arabinosylation of lipid A of SH7426 appears likely to be responsible for this resistance to the cationic,

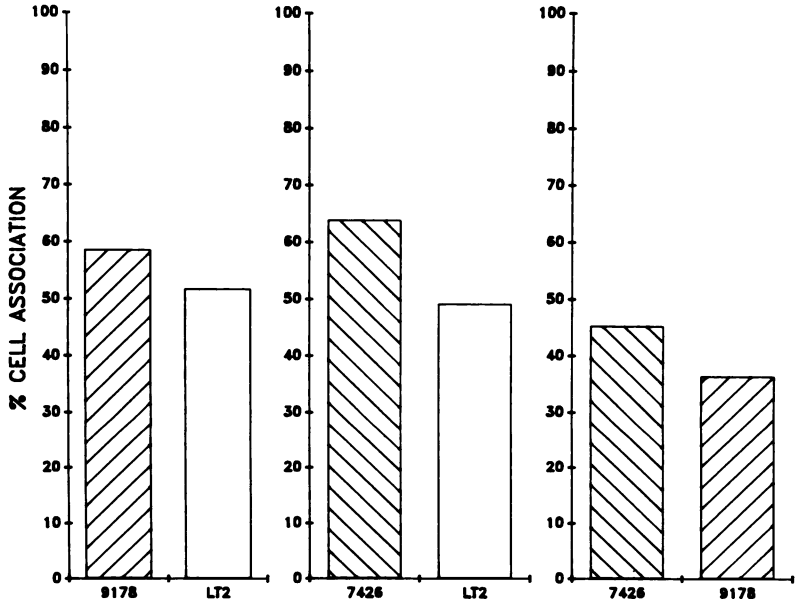

FIG. 6. Comparison of the phagocytosis of the bacteria in each experiment. The mean percentage of cell association plus or minus standard deviation in each case was as follows. Experiment 1: SH9178 (▨), 58.6 ± 14.4; LT2 (□), 51.8 ± 9.4; experiment 2: LT2, 49.2 ± 13.9; SH7426 (▧) 63.9 ± 9.7; experiment 3: SH9178, 36.4 ± 2.6, SH7426, 45.3 ± 6.6.

antimicrobial granule proteins (11), as well as to the cationic, amphipathic antibiotic polymyxin B. The results we present here strongly suggest that this change in arabinosylation of lipid A can endow the organism (SH7426) with a greater capacity to survive within neutrophils, compared with that of its parent, in spite of the fact that both are rough organisms. SH7426 became almost as resistant as LT-2. It may be inferred that the survival is due to resistance to the cationic antimicrobial proteins.

While doing this, we have also confirmed the earlier finding of Okamura and Spitznagel (6) that the length of LPS also increases the ability of the organism to survive within the PMN. This was shown by the greater intraleukocytic survival of LT-2 as compared with rough organisms.

In earlier communications, we have proposed that the greater resistance of SH7426 was due to the decrease in free PO_4^{-3} groups because of their being substituted with 4-aminoarabinose and conversion to zwitterions. This may indeed be the reason less cationic antimicrobial protein and less polymyxin B bind to the mutant bacteria and may be the reason they are more resistant to these agents. However, it is also true that hydrophobic interactions between the antimicrobial proteins and the bacteria are very important (3). 4-Aminoarabinose probably increases the hydrophilicity of the bacterial surface where it attaches. This would most likely be due to the hydrophilicity of the pentose moiety itself. It remains to be seen if this hydrophilic modification of lipid A is partly or even entirely responsible for the decreased antimicrobial action.

The studies presented here compare the survival of parent and mutant strains of bacteria in an environment as nearly similar as possible, since the comparisons were carried out within the same monolayers of PMN and probably, with high frequency, the same PMN.

The *Salmonella* strains used in this study have previously been shown to have quantitatively different resistances to the actions of cationic, antimicrobial granule proteins of PMN, as well as to the antibiotic polymyxin B (3, 11). The order of sensitivity to killing by normal PMN and by purified CAP57 was similar. Furthermore, when the ability of PMN to mount an oxidative burst was limited with sphingosine, the same order of sensitivity was observed, suggesting that much of the killing was oxygen independent. Sphingosine was used in this study as a surrogate for anaerobicity. We have compared the effect of sphingosine-treated, anaerobic and aerobic neutrophils and have found that sphingosine-treated neutrophils have bactericidal activities similar to that of anaerobic PMN (Stinavage and Spitznagel, in press). While sphingosine probably should not be considered a complete substitute for anaerobicity, we have found that sphingosine can be used as a convenient screening reagent for sensitivity to oxygen-independent killing mechanisms.

These studies also support the work of Rest et al. (7), who studied the role of the outer membrane of *S. typhimurium* in the resistance of the organisms to antimicrobial PMN extracts. We found that the fully smooth LT-2 was the most resistant to the antimicrobial actions of intact PMN. Furthermore, these studies extend the work of Rest and Spitznagel (8), who, using a myeloperoxidase-Cl^--H_2O_2 bactericidal system, found that the differences in sensitivity between rough and smooth organisms seen with oxygen-independent mechanisms were lost in the presence of the myeloperoxidase-Cl^--H_2O_2 system.

In summary, we have shown that the *pmrA* mutation that confers resistance to the granule proteins of PMN also greatly enhances the ability of these organisms to survive within intact, normal PMN that still possess their full bactericidal activity. Resistance of *S. typhimurium* to killing by PMN is influenced not only by the length of hydrophilic sugars of LPS which may hide binding sites for bactericidal substances but also by changes that affect the binding sites themselves, some of which at least appear to be on lipid A.

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