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Lipid A and Resistance of *Salmonella typhimurium* to Antimicrobial Granule Proteins of Human Neutrophil Granulocytes

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Granule extracts from human polymorphonuclear leukocytes were prepared and fractionated by chromatography on Sephadex G75-SF. One fraction exhibited potent antimicrobial activity against an Rd_1 lipopolysaccharide (LPS) mutant of *Salmonella typhimurium*. Susceptibility of the mutant to antimicrobial activity appeared to be due to binding of granule proteins to lipid A because isolated native LPS succeeded in blocking the antimicrobial activity of granule extracts whereas base-hydrolyzed LPS failed to do so. Centrifugation of control and base-hydrolyzed LPS-protein mixtures in cesium chloride gradients suggested that only control LPS formed complexes with antimicrobial proteins. Further evidence that bactericidal proteins from polymorphonuclear leukocyte granules interact with lipid A was that sublethal concentrations of polymyxin B (an antibiotic known to bind to lipid A) rendered target bacteria phenotypically resistant to granule proteins. Moreover, a mutant of *S. typhimurium* which synthesized a lipid A with decreased electronegativity due to increased 4-amino-4-deoxy-L-arabinosylation at the 4′-phosphate exhibited increased resistance to both polymyxin B and granule proteins. These results suggest that polymyxin B and antimicrobial proteins derived from polymorphonuclear leukocyte granules interact with lipid A in an analogous manner.

The bactericidal capacity of human neutrophil polymorphonuclear granulocytes (PMN) depends on oxidative as well as nonoxidative mechanisms. In fact, under certain conditions, nonoxidative (i.e., O_2-independent) systems may afford the principal antimicrobial activity available to PMN. For example, human PMN mediate killing under strictly anaerobic conditions (8, 14). PMN of patients with chronic granulomatous disease (15) are unable to reduce molecular O_2, but they kill *Neisseria gonorrhoeae* (18) and *Salmonella typhimurium* (25) as readily as do normal PMN. Mediators of human PMN O_2-independent killing of gram-negative bacteria include cationic proteins associated with the azurophilic granules (9, 16, 17, 23). Rest et al. (16, 17) studied the role of the *S. typhimurium* outer membrane in determining resistance to potent antimicrobial PMN granule extracts. The structure of the lipopolysaccharide (LPS) appeared to determine resistance. In a series of *S. typhimurium* outer membrane mutants placed in an isogenic background, resistance was directly related to the length of the carbohydrates in the O antigen and core oligosaccharide. Other evidence that LPS structure performed a direct role in determining resistance to granule protein was obtained by Modrzakowski and Spitznagel (9). They observed that purified LPS avidly bound several cationic proteins and blocked bactericidal activity.

Why did mutations which resulted in the synthesis of truncated LPS (16, 17, 22, 23) decrease resistance to granule proteins? One possibility was that, as the length of the carbohydrate chain diminished, differences in the phenotypic levels of resistance reflected progressively increased degrees of exposure of a heretofore undisclosed target site for binding of human cationic hydrophobic (9, 23) granule proteins. Lipid A, the anionic, hydrophobic head of the LPS molecule owing to its chemical properties and location in the outer membrane, could provide a possible binding site for these proteins. In this paper we show that the resistance of isogenic $PrmA^+$ and PrmA strains (20, 21) of *S. typhimurium* to the cationic, amphipatic antibiotic polymyxin B (PB) is similar to their resistance to the cationic granule proteins of PMN. The *prmA* mutation is known to cause increased 4-amino-4-deoxy-L-arabinose of the 4′-phosphate residue on the glucosaminyl disaccharide of lipid A. Hence, it is possible that cationic proteins are blocked from electrostatic binding to the phosphate due to an increased number of arbinosyl groups and, as a consequence, the resistance of the organism is increased.

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MATERIALS AND METHODS

Bacterial strains used in this study. *S. typhimurium* LT-2 and the isogenic LPS mutants which we used are listed in Table 1. The relevant LPS chemotype and source of these strains are also detailed. SH7426 is a PB-resistant mutant of SH9178. Expression of the *prmA* allele results in a four- to fivefold-increased substitution of 4-amino-4-deoxy-L-arabinose via ester linkage with a free phosphate residue in lipid A (20).

Preparation of PMN granule extracts. Granulocytes (kindly provided by R. Vogler, School of Medicine, Emory University) were obtained from a chronic myelocytic leukemia patient. The granulocyte concentrate consisted of promyelocytes (3%), myelocytes (14%), metamyelocytes (7%), band cells (31%), polymorphonuclear cells (40%), eosinophils (4%), and basophils (1%). Mixed granules were obtained from disrupted PMNs and extracted as previously described (9). Extracts in 0.2 M sodium acetate (pH 4.0) were concentrated at 4°C by ultrafiltration on a YM-5 membrane filter (Amicon Corp., Lexington, Mass.) and chromatographed on Sephadex G75-SF (Pharmacia. Fine Chemicals, Inc., Piscataway, N.J.) as described by Weiss et al. (23) to obtain a pool (termed fractionated antimicrobial material) with greatly enriched antimicrobial activity. Protein concentrations were determined as described by Bradford (3), using chicken egg white lysozyme as a standard.

TABLE 1. Bacterial strains used

S. typhimurium strain	LPS chemotype	Source/ reference
LT-2 (*prmA*$^+$)	Smooth	17
SL1004 (*prmA*$^+$)	Rd_1	17
SH9178 (*prmA*$^+$)	Rb	M. Vaara
SH7426 (*prmA*)	Rb	M. Vaara

Bactericidal activity of granule extracts. Overnight cultures of bacteria in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) were diluted in the same media and grown at 37°C to mid-logarithmic phase (optical density at 650 nm = 0.5). These cultures were washed two times with tryptone-saline (16) and serially diluted to provide 10^3 to 10^4 CFU/ml. Fractionated antimicrobial material (previously dialyzed against glass-distilled water) was serially diluted in a microtiter tray, using tryptone-saline, and sterilized by UV light. Bacteria (0.1 ml) were then added to each well. This mixture was incubated at 37°C for 1 h and then immediately placed onto Trypticase soy agar plates. The plates were incubated at 37°C for 24 h. All assays were performed in triplicate, and each data point is the average of at least three experiments. The variability of the results for each data point in each experiment was typically <5%. Bactericidal activity was calculated by the following equation: percent viability = number of CFU of surviving granule proteins per ml/number of CFU per milliliter incubated in the absence of granule proteins.

LPS inhibition of granule extracts. *Salmonella minnesota* R595 LPS (Re chemotype) prepared by the method of Galanos et al. (4) was kindly provided by D. C. Morrison (Emory University). This LPS was dissolved in pyrogen-free water to afford a final concentration of 2 mg/ml. The solution was clarified by sonication. Removal of ester-linked fatty acids and phosphates by mild alkaline hydrolysis (13) was accomplished as described by Morrison and Jacobs (10). Both base-hydrolyzed and control LPS were extensively dialyzed against sterile, glass-distilled water at 4°C. Various concentrations of these LPS preparations were incubated with 10 μg of fractionated antimicrobial material per ml for 30 min at room temperature and then exposed to 10^3 CFU of *S. typhimurium* SL1004 per ml.

Formation of LPS-protein complexes was assessed by buoyant density gradient centrifugation in cesium chloride (CsCl) as described by Morrison and Jacobs (10). Briefly, 500 μg of LPS (control and base hydrolyzed) was reacted with 500 μg of PB, poly-L-lysine, or fractionated antimicrobial material. These mixtures were then added to CsCl and 0.1 M Tris-hydrochloride (pH 8.1) to allow a final concentration of CsCl of 35.8%. Such gradients (5 ml) were centrifuged at 42,000 rpm in an SW50.1 rotor for 48 h at 4°C. Gradients were fractionated, and LPS was detected by the standard colorimetric assay for 2-ketodeoxyoctonic acid (26). CsCl densities were calculated from refractive index measurements, using an Abbe refractometer (American Optical Corp., Buffalo, N.Y.). All values were corrected for buffer refraction. PB sulfate and poly-L-lysine (6,000 molecular weight) were purchased from Sigma Chemical Co., St. Louis, Mo.

PB inhibition of PMN granule extracts. PB was resuspended in pyrogen-free water to afford a final concentration of 2 mg/ml. Increasing amounts of PB were added to 10^3 CFU of *S. typhimurium* SL1004 per ml, and the mixture was incubated at room temperature for 30 min. After this incubation the mixture was exposed to 5 μg of fractionated antimicrobial protein per ml. Incubation of this reaction was as for the standard bactericidal assay.

RESULTS

Modrzakowski and Spitznagel (9) determined that rough and smooth LPS effectively blocked the antimicrobial activity of both crude and fractionated human PMN granule extracts. Their results suggested that complexes had formed between the LPS and the proteins. To test this more directly, we examined whether the buoyant density of LPS would change in the presence of cationic granule proteins. The buoyant density of R595 LPS incubated with cationic granule proteins changed from 1.36 to 1.30 g/cm^3 (Table 2). This change was substantial, but less than the change seen when LPS was incubated with PB; LPS-PB mixtures exhibited a buoyant density of 1.24 g/cm^3. It was similar, however, to the change caused by poly-L-lysine (density, 1.30 g/cm^3).

These results suggested complex formation. Were the complexes formed due to interaction at the sites that bind PB? To test this, mild base hydrolysis of R595 LPS, previously shown by Neter et al. (13) to remove ester-linked phosphate and fatty acid groups of lipid A, was used to ascertain whether binding of granule proteins would be abrogated in the same manner as described for the interaction of lipid A and PB (10). Base-hydrolyzed LPS exhibited a buoyant density in CsCl of 1.48 g/cm^3 (Table 2). However, incubation of this LPS with cationic proteins failed to result in a change in the buoyant density of hydrolyzed LPS.

The hydrolyzed LPS failed (Fig. 1) to inhibit the antimicrobial action of the granule protein preparation, although the native LPS inhibited this readily. Inhibition of bactericidal activity by native LPS was dose dependent and complete. These results suggested that the complexes were formed with the ester-linked phosphate or ester-linked fatty acid group or both. The failure of hydrolyzed LPS to perform this inhibition suggested that the base-sensitive lipid A, ester-linked constituents were required for binding the antimicrobial components.

PB has been shown to bind lipid A due to both ionic and hydrophobic interactions (10). Since our results suggested that PB (10) and cationic proteins formed stable complexes with LPS, we asked whether they might interact with the same or similar cell surface receptors. To test this, we preincubated strain SL1004 with increasing but sublethal concentrations of PB and then measured the sensitivity of PB-treated bacteria to antimicrobial granule protein preparations. The data (Fig. 2) demonstrated that prior incubation of the target bacteria with PB rendered them phenotypically resistant to the antimicrobial preparation.

That prior incubation of target bacteria with PB resulted in their increased resistance to the bactericidal activity of a

TABLE 2. Modification of the CsCl buoyant density of R595 LPS due to addition of cationic granule proteins

LPS prepn	Buoyant density (g/cm^3)
R595 LPS alone	1.36
R595 LPS + cationic granule proteins	1.30
R595 LPS + PB	1.24
R595 LPS + poly-L-lysine	1.30
Base-hydrolyzed R595 LPS	1.48
Base-hydrolyzed R595 LPS + cationic granule protein	1.48

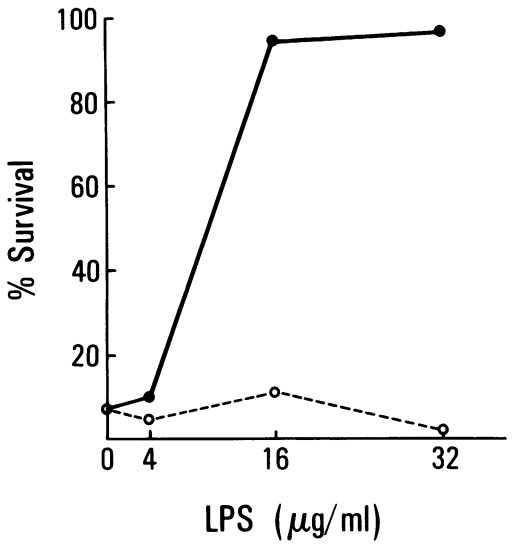

FIG. 1. Inhibition of antimicrobial activity of fractionated granule proteins by LPS: effect of mild alkaline hydrolysis of R595 LPS. Control and base-hydrolyzed LPS preparations were reacted with fractionated, antimicrobial protein for 30 min at 22°C and then added to 0.1 ml of strain SL1004 (10^3 CFU/ml). The final concentration of granule protein was 10 μg/ml; that of LPS ranged from 4 to 32 μg/ml. Symbols: ●, percent survival of strain SL1004 exposed to control LPS-granule protein mixture; ○, percent survival of strain SL1004 exposed to base-hydrolyzed LPS-granule protein mixture.

granule antimicrobial preparation suggested that these proteins and PB had reacted with similar cell receptors. This hypothesis was further examined by the use of an isogenic pair of *S. typhimurium* which differed only at a locus (*prmA*$^+$) involved in resistance to PB. The results (Fig. 3) demonstrated that strain SH7426 (*prmA*) was considerably more resistant to the antimicrobial preparation than the parental strain SH9178 (*prmA*$^+$). Thus, the data suggest that a mutation which affects lipid A structure results not only in increased resistance to PB, but also in PMN granule proteins.

DISCUSSION

The O_2-independent antimicrobial mediators of human PMN have attracted renewed attention in recent years due to the observation that PMNs unable to mount an oxidative response readily killed certain species of gram-negative bacteria (18, 25). The importance of these observations was recently confirmed in studies which examined phagocytic killing in the absence of molecular O_2; human PMN readily killed wild-type and LPS mutants of *S. typhimurium* under anaerobic conditions (14). Moreover, they did this in an ordered fashion, suggesting the same effects seen (16, 17) with cationic granule proteins in vitro with bacterial suspensions.

Several workers have examined the environmental factors (16, 17), bacterial cell surface structures (9, 16, 17, 23), and human PMN granule components (9, 17, 23) necessary for bactericidal activity. Earlier studies (17) suggested that, although binding of bactericidal proteins was temperature independent, antimicrobial activity was temperature dependent. These results suggested a two-step process and implied that binding of cationic granule proteins could be experimentally distinguished from their bactericidal activity.

In studies on the gram-negative bacterial cell surface structures involved in binding antimicrobial cationic proteins, Rest et al. (16, 17) showed a direct role for LPS in determining resistance to granule extracts. Resistance was ordered and directly proportional to the length of the core oligosaccharides. However, these mutants probably also differed in the qualitative and quantitative distribution of several outer membrane proteins (1). Accordingly, Modrzakowski and Spitznagel (9) reexamined the resistance of such LPS mutants and found that the phenotypic differences in granule extract sensitivity was indeed ordered. Moreover, extracted LPS effectively blocked biological activity. However, weight for weight, wild-type or smooth LPS was more active in inhibiting antimicrobial cationic protein than was the rough (Re) LPS. The LPS-cationic protein complexes were analyzed by gel electrophoresis, and the results suggested that several cationic proteins were avidly bound to the LPS.

We have continued to study the bacterial cell surface structural requirements necessary for reactions with antimicrobial substances present in PMN granules. We hypothesized that the earlier observations (9, 16, 17, 23) relating decreases in the phenotypic levels of resistance to human granule extracts as a function of LPS roughness might in part be due to increased exposure in rough mutants of lipid A to the granule proteins.

The known interaction (5, 10–12, 20, 21) of the cationic antibiotic PB with lipid A suggested that cationic granule proteins might bind to lipid A in an analogous manner. Three lines of evidence suggested this. Chemical modification of LPS by mild alkaline hydrolysis selectively hydrolyzed the

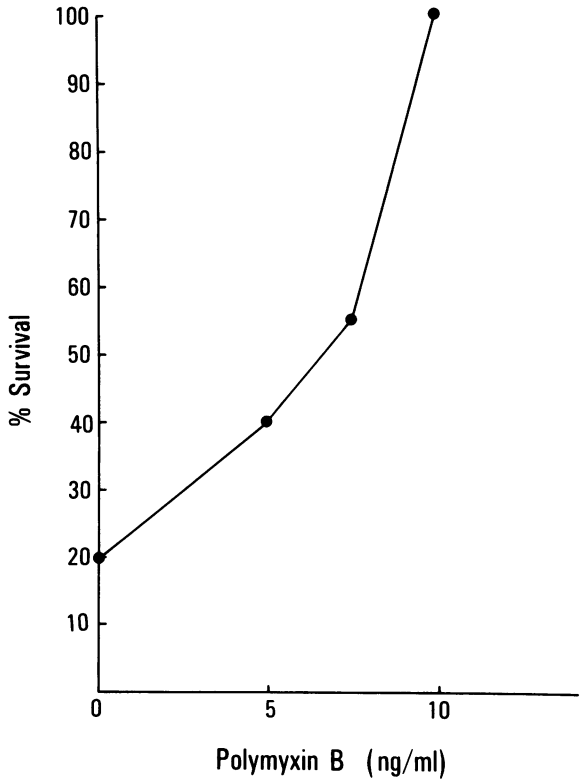

FIG. 2. Inhibition of granule protein antimicrobial activity by PB. Strain SL1004 was pretreated with increasing but sublethal concentrations of PB (final concentrations, 5 to 10 ng/ml) and then exposed to granule proteins (5 μg/ml).

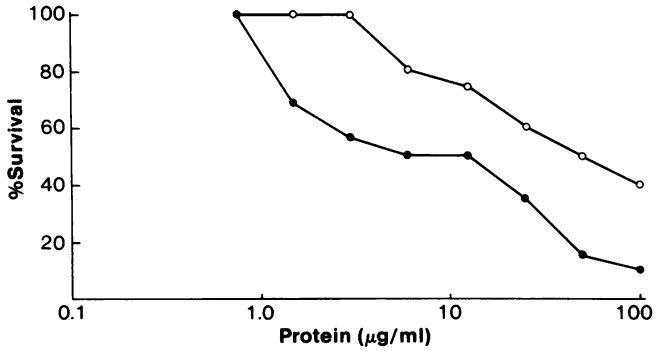

FIG. 3. Increased resistance of strain SH7426 to granule proteins due to a mutation (*prmA*) affecting PB resistance and lipid A structure. Symbols: ●, percent survival of strain SH9178 (*prmA*$^+$); ○, percent survival of strain SH7426 (*prmA*).

lipid A ester-linked fatty acids and phosphates (13) and resulted in decreased binding of PB (10). Similar hydrolysis of R595 LPS abrogated the ability of that LPS to block the bactericidal activity of granule extracts (Fig. 1). preincubation of an Rd_1 LPS mutant (SL1004) with sublethal concentrations of PB resulted in phenotypic resistance to the antimicrobial granule protein preparation (Fig. 2). Finally, we found that a mutation (*prmA*) which dictated increased PB resistance likewise dictated increase resistance to granule proteins (Fig. 3).

The *prmA* mutation has been extensively studied by Vaara et al. (20, 21). Expression of *prmA* results in the chemical modification of lipid A. This modification involves increased (four- to fivefold) substitution of 4-amino-4-deoxy-L-arabinose at a phosphate only partially (10 to 15%) substituted with 4-amino-4-deoxy-L-arabinose in the wild-type *S. typhimurium* in lipid A. Presumably, this increased degree of substitution reduced the electronegativity of the lipid A. This caused decreased binding of cationic substances (21). Whether the binding of cationic granule proteins mediates antimicrobial activity by a mechanism involving membrane disorganization (as has been proposed for PB [2, 6, 7, 19]) cannot be ascertained with our data.

Both ionic and hydrophobic bonds are involved in the interaction of PB and lipid A. Our data suggest that similar interactions occur between human cationic PMN granule proteins and lipid A. Ionic bonding is indicated by our observation that a mutation (*prmA*) which decreases lipid A electronegativity resulted in increased resistance to granule proteins. Hydrophobic interaction between lipid A and granule proteins is suggested by our observation that R595 LPS and granule proteins maintained stable complexes even in high salt (3.4 M CsCl). Indeed, a similar proposal for the stability of LPS-PB complexes in CsCl has been put forth by Morrison and Jacobs (10). Our suggestion that hydrophobic and ionic bonds are involved in granule proteins and lipid A interaction is consistent with the recent results of Weiss et al. (24). They studied the interaction of the cationic bactericidal/permeability-increasing protein derived from rabbit PMN. Their data demonstrated that the initial interaction of rabbit bactericidal/permeability-increasing protein with the gram-negative outer membrane is mediated by ionic bonds and is subsequently followed by hydrophobic interactions.

The use of well-defined mutants which differ only at the *prmA*$^+$ locus in studies with purified cationic proteins should provide additional insights regarding the molecular mechanisms which result in nonoxidative killing of *S. typhimurium*. At present, we have no data regarding the nature of the antimicrobial protein(s) used in this study. Earlier work by Weiss et al. (23) and Modrzakowski and Spitznagel (9) suggested that the antimicrobial fraction used in this study comprises two antimicrobial cationic proteins which differ in apparent molecular weight. Whether these proteins interacted with the same or different cell surface receptors remains to be determined. Whether our results dealing with LPS mutants of *S. typhimurium* can be extended to other gram-negative bacteria is uncertain; several cationic proteins might exhibit antimicrobial activity and different receptors might be important in binding such toxic substances.

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