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Killing of *Proteus mirabilis* by Polymorphonuclear Leukocyte Granule Proteins: Evidence for Species Specificity by Antimicrobial Proteins

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Low-molecular-weight (M_r, ca. 3,800) polypeptides containing human defensins HNP-1 and HNP-2 (T. Ganz, M. S. Selsted, D. Szlarek, S. L. Harwig, K. Daher, D. F. Bainton, and R. I. Lehrer, J. Clin. Invest. 76:1427–1434, 1985) prepared in our laboratory from acid extracts of human polymorphonuclear granulocyte granules and purified human defensins were found to exert potent bactericidal action against *Proteus mirabilis*. The antimicrobial action of the extracts of human polymorphonuclear leukocyte granules against *P. mirabilis* appears to be due to the presence of the defensins. Because *P. mirabilis* resists the antimicrobial action of other granule proteins, we interpret the present results to mean that the various antimicrobial proteins display species specificity in their microbicidal action.

We have recently described the purification of two antimicrobial proteins from acid extracts of human polymorphonuclear leukocyte (PMN) granules. These proteins, CAP37 and CAP57, were found to exhibit potent but selective bactericidal activity against a variety of gram-negative bacteria (7). Two lines of evidence suggest that these proteins bind to the 4′ lipid A phosphate (5–7), or phosphates in the inner core region of lipopolysaccharide (LPS), or both. First, mutations that decrease the type and amount of sugars in the core oligosaccharide or the presence of O antigen (or both) increase bacterial susceptibility to CAP37 and CAP57. Second, a mutation (*pmrA*) that increases the substitution of 4′-amino-1-deoxyarabinose (4-ARN) at the 4′ phosphate (10) decreases the susceptibility of *Salmonella typhimurium* to CAP37 and CAP57.

Our hypothesis that the availability of electronegative residues provided by the 4′ phosphate of lipid A determines microbial susceptibility to cationic antimicrobial proteins predicts that bacteria that produce a lipid A completely substituted by 4-ARN should be highly resistant to CAP57 and CAP37. Indeed, we have found that CAP37 and CAP57 were ineffectual against fully smooth and deep rough strains of *Proteus mirabilis* (7; Shafer et al., unpublished observations) known to produce a lipid A molecule completely substituted by 4-ARN at the 4′ phosphate (9).

Although *P. mirabilis* resists killing by CAP37 and CAP57, it is nevertheless susceptible, in vitro, to extracts of human PMN granules. This suggests that within granules, antimicrobial proteins other than CAP57 and CAP37 exert antimicrobial activity against *P. mirabilis*. Accordingly, we sought to identify the proteins responsible for such action.

MATERIALS AND METHODS

Granulocyte concentrates (>95% PMNs) were obtained by leukapheresis from two healthy donors at Emory University Hospital. Granules were collected from homogenized PMNs and extracted with 0.2 M sodium acetate (pH 4.0) as described by Rest et al. (4). The crude granule extract was concentrated by ultrafiltration (6) and applied to a Sephadex G-75 (Pharmacia Fine Chemicals, Piscataway, N.J.) column as described previously (8). Fractions were pooled and stored at 4°C before bactericidal assays (see below). The pooled protein exhibiting bactericidal activity was dialyzed against 1% (vol/vol) acetic acid by ultrafiltration by using an Amicon YM-u2 membrane in a stirred cell (Amicon Corp., Lexington, Mass.). The protein was lyophilized overnight and dissolved in 0.05 M sodium phosphate buffer (pH 7.0) containing 10% (vol/vol) acetonitrile (buffer A). Particulate material was removed by centrifugation at 10,000 × *g* for 5 min. Protein (500 μg) was applied to a Bio-Sil TSK-CM-3-SW ion-exchange high-performance liquid chromatography column (Bio-Rad Laboratories, Richmond, Calif.). Bound protein was eluted with a linear gradient of buffer A and buffer A–0.1 M sodium chloride. Fractions were lyophilized and dissolved in distilled water before use. Protein concentrations were determined as described by Bradford (1). The purity of the bactericidal proteins was estimated by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (17.5% [wt/vol] polyacrylamide) profiles as described by Shafer et al. (6).

Isogenic *P. mirabilis* 1959S (smooth LPS chemotype) and Re_{45} (Re LPS chemotype) were kindly provided by D. C. Morrison (Department of Microbiology, University of Kansas School of Medicine). Bactericidal assays were conducted by using tryptone broth (pH 7.2) essentially as described by Rest et al. (4).

RESULTS

Rest et al. (4) showed that crude granule extracts killed deep rough LPS mutants more readily than they did fully smooth bacteria. We adopted a similar approach to detect proteins in crude granule extracts having the capacity to exert bactericidal activity against *P. mirabilis*. Accordingly, we tested the resistance to killing by crude granule extracts of a smooth strain (1959S) and a Re mutant (Re_{45}) of *P. mirabilis* (9). Strain Re_{45} was substantially less resistant than strain 1959S to crude granule extracts (Fig. 1). The approximate 50% effective dose (ED_{50}) of the crude granule extract against Re_{45} was 125 μg of protein per ml, whereas ca. 350 μg of protein per ml was the ED_{50} against strain 1959S. We

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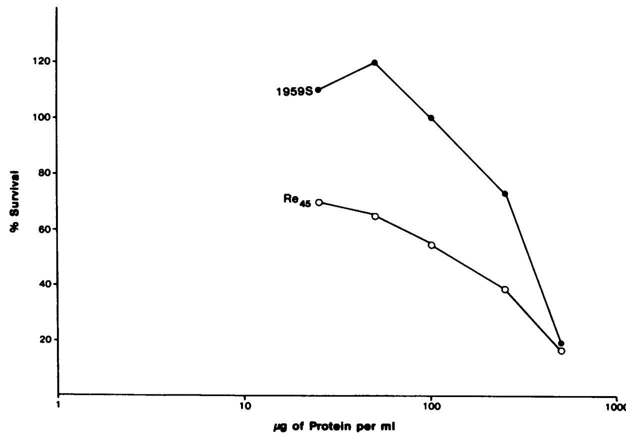

FIG. 1. Resistance of *P. mirabilis* to crude granule extracts is decreased by synthesis of Re LPS. Levels of susceptibility of isogenic strains 1959S (smooth LPS; ●) and Re_{45} (Re LPS; ○) to the lethal action of crude granule extracts were determined. The Re LPS mutant was found to be at least threefold less resistant than the parental smooth strain (1959S).

therefore used strain Re_{45} in subsequent antimicrobial assays to detect granule proteins other than CAP37 and CAP57 having the capacity to exert antibacterial activity against *P. mirabilis*.

To obtain the proteins responsible for killing *P. mirabilis*, a crude granule extract containing 300 mg of protein was fractionated by molecular-sieve chromatography by using Sephadex G-75. The resulting chromatogram is presented in Fig. 2. Column fractions were pooled (pools A to H) as shown and tested for antimicrobial activity against strain Re_{45}. When tested in antimicrobial assays that made use of 125 µg of protein per ml, only pool H, containing proteins with an apparent M_r greater than or equal to that of lysozyme (14 kilodaltons; pool F), appeared to have the capacity to exert antimicrobial activity (data not shown). The approximate ED_{50} of the protein in pool H was 25 µg/ml, or ca. fivefold less than that of the crude granule extract (Fig. 1). CAP57 and CAP37 were present in pool B (data not presented), yet this pool failed to kill *P. mirabilis* ($ED_{50} > 125$ µg/ml), despite having the capacity to kill *S. typhimurium* ($ED_{50} = 5$ µg/ml). By using the test conditions described, other gram-negative bacteria producing either smooth or deep rough LPS (*S. typhimurium* and *Neisseria gonorrhoeae*) resisted the bactericidal action of the proteins (125 µg/ml) in pool H capable of killing *P. mirabilis* (data not presented).

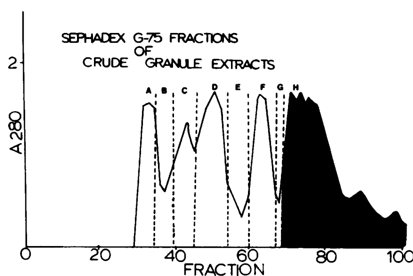

FIG. 2. The antimicrobial action of crude granule extracts against *P. mirabilis* is restricted to granule proteins with a molecular weight, as determined by gel exclusion chromatography, less than that of lysozyme. Shown is the Sephadex G-75 chromatographic profile of fractionated granule extracts. PMN lysozyme was localized in pool F, whereas the proteins exerting bactericidal action against *P. mirabilis* were detected in pool H (darkened area).

TABLE 1. Bactericidal activity of low-molecular-weight proteins obtained by high-performance liquid chromatography

Fraction no.	% Survival of *P. mirabilis*[a]
15	78.3 ± 3.1
18	38.1 ± 4.3
19	46.3 ± 4.0
20	59.4 ± 3.3

[a] Determined by using 20 µg of protein per ml. Values reported are the average of three determinations (± standard deviation).

To obtain greater purification of proteins exerting antibacterial effects against strain Re_{45}, the protein in pool H was dialyzed against 1% acetic acid, lyophilized, dissolved in 0.05 M sodium phosphate (pH 7.0) containing 10% (vol/vol) acetonitrile, and applied to a Bio-Sil TSK-CM-3-SW column. The resulting chromatogram indicated the presence of at least three distinct species. Column fractions (fractions 15, 18, 19, and 20) were individually dialyzed against 0.1% acetic acid, lyophilized, and then dissolved in distilled water. The dissolved proteins were tested for antibacterial effects against strain Re_{45}. The results (Table 1) showed that at the tested concentration of protein, the protein present in fractions 18 and 19 exerted greater antimicrobial effects than the protein in fractions 15 and 20. When analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the approximate M_r of the antimicrobial proteins (fractions 15, 18, 19, and 20) was estimated to be 3,800 (data not presented).

Our finding that the anti-*Proteus* protein(s) exhibited an approximate M_r of 3,800 suggested that it might be similar to the defensins described by Ganz et al. (2). Accordingly, we provided this group with high-performance liquid chromatography column fractions that exhibited bactericidal activity against strain Re_{45}. By using acid-urea gel electrophoresis, the presence of cathode-migrating HNP-1 and -2 was detected, as well as minute amounts of at least one other granule protein (M. Selsted, personal communication).

Purified HNP-1, HNP-2, and HNP-3 (kindly supplied by M. Selsted) were tested for bactericidal activity against *P. mirabilis*. HNP-1, but not HNP-2 or -3, exerted measurable bactericidal action against *P. mirabilis* after 60 min at 37°C (Table 2). However, after 120 min all three defensins demonstrated significant bactericidal action against *P. mirabilis*.

DISCUSSION

Because of their high concentration in granule extracts (ca. 25% of the total protein content), the defensins have been suggested to contribute significantly to O_2-independent killing of bacteria by PMNs (2, 3). Our results suggest that

TABLE 2. Antimicrobial action of purified human defensins against *P. mirabilis*

Defensin	ED_{50} (µg/ml) at:	
	1 h	2 h[a]
HNP-1	25	12.5
HNP-2	>50	12.5
HNP-3	>50	25

[a] The observed time-dependent killing of *P. mirabilis* by HNPs is consistent with the recent work of Greenwald and Ganz (3).

they have the capacity to kill *P. mirabilis* and are responsible for the bactericidal capacity of granule extracts when such extracts are tested against this bacterium. This is consistent with our finding that on a weight basis, 10-fold less purified HNP-1 and HNP-2 was required to achieve an ED_{50} (12.5 μg/ml) as compared with that of the crude granule extract (ED_{50} = 125 μg/ml). Moreover, neither purified CAP37 or CAP57 in our in vitro antimicrobial assay kills *P. mirabilis* at this or higher protein concentrations (7; Shafer et al., unpublished observations). We interpret this to mean that granule proteins exert selective antimicrobial action, and this is likely due to the chemical composition of the bacterial cell surface.

The concept that granule proteins exhibit selective bactericidal action is consistent with the earlier work of Zeya and Spitznagel (11) and the more recent studies of Greenwald and Ganz (3). The former showed that the cationic, antimicrobial, arginine-rich granule proteins prepared from rabbit PMNs, now known as defensins (2), exhibited different degrees of antimicrobial activity against a range of bacteria, whereas the latter group suggested that the bactericidal capacity of crude extracts, known to contain several antimicrobial proteins, against *Acinetobacter calcoaceticus* was due to the action of defensins.

Against gram-negative bacteria, this antimicrobial specificity appears to be dictated at least in part by the extent of 4-ARN substitution at the 4′ lipid A phosphate residue at the nonreducing glucosaminyl residue. Hence, *P. mirabilis* producing a lipid A molecule completely substituted by 4-ARN at the 4′ phosphate (9) resists the antimicrobial action of CAP57 and CAP37 but is killed by the protein in pool H (Fig. 2) containing defensins at concentrations that are ineffectual under our test conditions against *S. typhimurium*, *Escherichia coli*, and *N. gonorrhoeae*. The antimicrobial species specificities of these granule proteins may, in part, explain the capacity of PMNs to kill a variety of pathogens.

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LITERATURE CITED

1. **Bradford, M. M.** 1976. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
2. **Ganz, T., M. S. Selsted, D. Szlarek, S. L. Harwig, K. Daher, D. F. Bainton, and R. I. Lehrer.** 1985. Defensins: natural peptide antibiotics of human neutrophils. J. Clin. Invest. **76:**1427–1434.
3. **Greenwald, G. I., and T. Ganz.** 1987. Defensins mediate the microbicidal activity of human neutrophil granule extract against *Acinetobacter calcoaceticus*. Infect. Immun. **55:**1365–1368.
4. **Rest, R. F., M. H. Cooney, and J. K. Spitznagel.** 1977. Susceptibility of lipopolysaccharide mutants to the bactericidal action of human neutrophil lysosomal fractions. Infect. Immun. **16:** 145–151.
5. **Shafer, W. M., S. G. Casey, and J. K. Spitznagel.** 1984. Lipid A and resistance of *Salmonella typhimurium* to antimicrobial granule proteins of human neutrophil granulocytes. Infect. Immun. **43:**834–838.
6. **Shafer, W. M., L. E. Martin, and J. K. Spitznagel.** 1984. Cationic antimicrobial proteins isolated from human neutrophil granulocytes in the presence of diisopropyl fluorophosphate. Infect. Immun. **45:**29–35.
7. **Shafer, W. M., L. E. Martin, and J. K. Spitznagel.** 1986. Late intraphagosomal hydrogen ion concentration favors the in vitro antimicrobial capacity of a 37-kilodalton cationic granule protein of human neutrophil granulocytes. Infect. Immun. **53:**651–655.
8. **Shafer, W. M., V. C. Onunka, and P. J. Hitchcock.** 1986. A spontaneous mutant of *Neisseria gonorrhoeae* with decreased resistance to human neutrophil granule proteins. J. Infect. Dis. **153:**910–917.
9. **Sidorczyk, Z., U. Zahringer, and E. T. Rietschel.** 1983. Chemical structure of the lipid A component of the lipopolysaccharide from a *Proteus mirabilis* Re mutant. Eur. J. Biochem. **137:** 15–22.
10. **Vaara, M., T. Vaara, M. Jensen, I. Helander, M. Nurimen, E. T. Reitschel, and P. H. Makela.** 1981. Characterization of the lipopolysaccharide from the polymyxin-resistant mutants of *Salmonella typhimurium*. FEBS Lett. **129:**145–149.
11. **Zeya, H. I., and J. K. Spitznagel.** 1968. Arginine-rich proteins of polymorphonuclear leukocyte lysosomes. J. Exp. Med. **127:** 927–941.