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Bactericidal Activity of Fractionated Granule Contents from Human Polymorphonuclear Leukocytes

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Proteins from human polymorphonuclear leukocyte granules were extracted with 0.2 M acetate, pH 4.0, and fractionated by Sephadex G-100 column chromatography. The fractions demonstrated selective bactericidal action against a deep rough cell wall mutant of *Escherichia coli* O111:B4 with rough lipopolysaccharide and cell wall mutants of *Salmonella typhimurium* LT-2 with lipopolysaccharide of Ra, Rc, Rd_1, Rd_2, and Re types. Smooth parent strains were most resistant to the bactericidal action. Fractions with greatest activity for the mutants were from valley regions (regions of low protein concentration) between three high protein peaks comprising myeloperoxidase, protease, and lysozyme, respectively. Susceptibility of the mutants to bactericidal action increased as sugar residues decreased in lipopolysaccharide. Gram-positive bacteria were susceptible to different fractions than were the gram-negative bacteria.

Polymorphonuclear leukocytes (PMN) possess a variety of potentially antimicrobial substances and hydrolytic enzymes distributed within their cytoplasmic granules. Neutrophil bactericidal mechanisms can be divided into two groups, those mechanisms dependent upon oxidative processes such as the myeloperoxidase-Cl^--H_2O_2 system (5) and O_2, and those independent of oxidative processes, e.g., lysozyme, cationic proteins, and apolactoferrin (27). Whereas oxidative processes depend, at least in part, on cytosol-localized soluble enzymes and cofactors, the nonoxidative processes appear to be dependent upon proteins wholly localized within the neutrophil granules. Approaches to identifying and defining the mechanism of these bactericidal substances have involved the utilization of neutrophil extracts (3, 7), neutrophil granule extracts (8, 28), and purified granule proteins (4, 5, 10, 12, 19, 25, 28) added to test microorganisms in vitro. Rest et al. (21) showed by using smooth *Enterobacteriaceae* and their lipopolysaccharide (LPS)-deficient mutants that a series of deep rough mutants had an ordered increase in sensitivity to human neutrophil lysosomal fractions inversely related to the length of their LPS carbohydrate chains. Using *Salmonella typhimurium* LT-2 and a series of progressively rough mutant strains, they showed that azurophil granule extracts were much more bacteriostatic and bactericidal than were specific granule extracts (22).

The parent and mutant strains possess different substrates on their surface and as such can be used as probes for detecting and defining different enzyme systems involved in the bactericidal process. In the present report, we utilized the sensitivity of deep rough mutants of *Salmonella typhimurium* LT-2 to detect bactericidal fractions obtained from the Sephadex G-100 column chromatographic separation of acetate-extracted human PMN granule protein. The sensitivity to these fractions of other gram-negative parent and LPS-deficient mutant strains, as well as selected gram-positive microorganisms, is also described.

MATERIALS AND METHODS

Purification of PMN. Human neutrophils were obtained in large quantity with leukophoresis from a patient with chronic granulocytic leukemia. These studies were approved by the Committee on the Protection of the Rights of Human Subjects at the University of North Carolina. Neutrophils were purified by dextran and Ficoll-Hypaque sedimentation as previously described (21, 23).

Purification of granules and preparation of granule extracts. Approximately 9.5×10^{10} neutrophils were recovered for homogenization. PMN were homogenized four times at 4°C in 0.34 M sucrose at 45-s intervals in a Porter-Blum homogenizer (19,000 rpm), followed by centrifugation at $126 \times g$ for 15 min at 4°C to remove cellular debris. The supernatant was then centrifuged at $20,000 \times g$ for 30 min at 4°C to pellet PMN granules. The pelleted granules were suspended in 100 ml of 0.2 M acetate buffer, pH 4.0, with 0.01 M $CaCl_2$ and extracted overnight at 4°C with gentle stirring. The extraction procedure was repeated twice, and acetate extracts were pooled and concentrated by ultrafiltration (Amicon UM-2 filter) before

Sephadex G-100 column chromatography. Approximately 300 mg of granule protein was chromatographed following previously published procedures (20). Fractions were pooled, concentrated by ultrafiltration (Amicon UM-05 filter) to 4 to 6 mg/ml, and dialyzed against phosphate-buffered saline, pH 7.0, before use in bactericidal assays.

Bacteria. *S. typhimurium* LT-2 and LPS-deficient mutants derived from it have been described previously (21, 26). *Escherichia coli* O111:B4 and its Re mutant RC59 were obtained from Loretta Leive of the National Institutes of Health (6, 14). *Streptococcus faecalis* F-24, *Staphylococcus albus*, and *Staphylococcus aureus* were laboratory-maintained cultures. Growth media and conditions have been described previously (21).

Bactericidal assays. Bactericidal assays were done by the method of Rest et al. (21, 22) in a total of 0.2 ml of tryptone–NaCl (pH 7.0) in the wells of a small plastic tray. Assay mixtures contained 2×10^3 colony-forming units of the appropriate bacteria per ml, and incubations were for 1 h at 37°C unless otherwise indicated. After incubation, 0.1 ml of the assay mixture was plated on Trypticase soy agar plates, and colony-forming units were counted at 18 to 24 h. Results are expressed as percentage of viable bacteria, where viable bacteria are defined as those bacteria that can produce colonies on agar. No clumping was observed in Gram-stained smears of the incubation mixtures.

Protein. Protein was measured by the method of Lowry et al. (9), with chicken egg white lysozyme as standard.

Materials. Trypticase soy broth and agar were from BBL. Tryptone was purchased from Difco Laboratories. All other chemicals were of reagent grade.

RESULTS

The elution profile obtained from the Sephadex G-100 column chromatographic separation of acetate-extracted human PMN granule protein is shown in Fig. 1. The profile was similar to that obtained from Sephadex G-75 separation described by others (15–17, 24) and showed four major protein peaks containing the bulk of myeloperoxidase (A) (24), neutral proteases (B) (16, 24), lysozyme (C) (18), and a fourth undefined protein peak (D). The four major protein areas and the valley portions (areas of low protein concentration) were concentrated and tested for bactericidal activity.

Susceptibility of smooth versus rough gram-negative bacteria. *E. coli* O111:B4 and its deep rough mutant RC59 were tested against 50 μg of each Sephadex G-100-pooled fraction. Figure 2 shows that the rough mutant was more sensitive to the bactericidal action of pooled fractions than was the smooth parent. The fractions showing the highest bactericidal activity against the rough mutant were valley AB, between high protein peaks of myeloperoxidase and protease, and valley CD, between high protein peaks of lysozyme and the undefined peak D. To determine the bactericidal activity of the Sephadex G-100 fractions against other gram-negative bacteria, *S. typhimurium* LT-2 and its deep rough mutant TA2168 were also tested. Figure 3 shows that the deep rough mutant TA2168 was sensitive to most of the Sephadex G-100 fractions, whereas the LT-2 smooth parent was virtually unaffected. The extreme sensitivity of the deep rough mutant to the fractionated human PMN granule contents prompted an examination of the effect of Sephadex fractions on *S. typhimurium* mutants of intermediate roughness. Figure 4 shows that peak A, the myeloperoxidase peak, demonstrated no bactericidal activity against any of the rough mutants as was the case with the deep rough TA2168 mutant. Valley AB protein had no effect on the LT-2 parent. However, all of the rough mutants proved to be extremely sensitive to the bacteri-

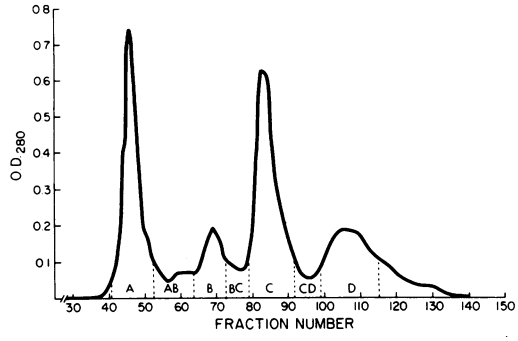

FIG. 1. *Elution profile obtained for Sephadex G-100 column chromatographic separation of human PMN granule protein. Approximately 300 mg was applied to the column (5 by 90 cm). Elution was with 0.2 M sodium acetate buffer, pH 4.0. Fraction volumes were 16.0 ml. Fractions 1 to 38 represent the void volume. Peak A represents the bulk of PMN myeloperoxidase (150,000 molecular weight). Peak C represents the PMN lysozyme (14,000 molecular weight). All operations were conducted at 4°C.*

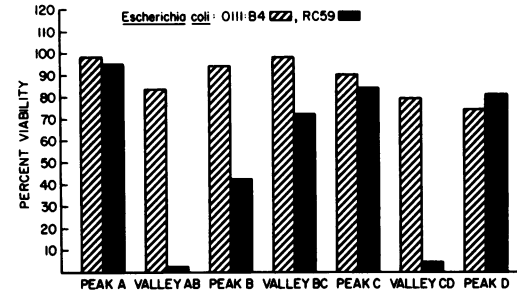

FIG. 2. *Effect of 50 μg of fractionated granule protein on the viability of E. coli O111:B4 and its rough mutant RC59.*

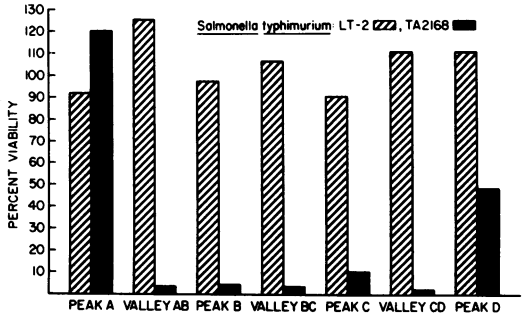

FIG. 3. *Effect of 50 μg of fractionated granule protein on the viability of S. typhimurium LT-2 and its rough LPS mutant TA2168.*

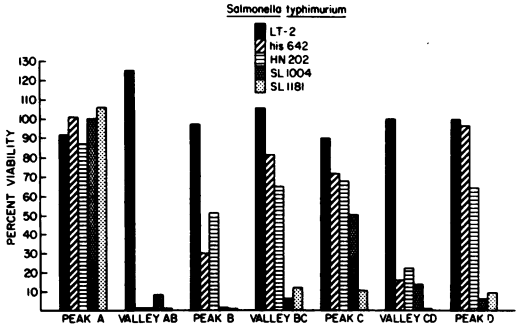

FIG. 4. *Effect of 50 μg of fractionated granule protein on the viability of S. typhimurium LT-2 and its LPS mutants: his 642 (Ra), HN202 (Rc), SL1004 (Rd_1), and SL1181 (Rd_2).*

cidal activity of this fraction. The viability of the his 642 and HN202 mutants was 31 and 52%, respectively, when treated with the peak B neutral proteases. The Rd_1-type mutant SL1004 and the Rd_2-type mutant SL1181 appeared to be extremely sensitive to the bactericidal activity of the peak B protein. Treatment of the smooth parent and rough mutant with valley BC protein or peak C protein showed increases in antimicrobial resistance of the rough mutants as the degree of smoothness increased. All of the rough mutants were sensitive to the action of valley CD protein, and the Rd-type rough mutants showed substantial sensitivity to the peak D protein. The high degree of bactericidal activity observed with valley AB protein could not be produced by mixing peak A and peak B protein, suggesting that a distinct protein species was functioning independently in the area of low protein concentration between the myeloperoxidase peak and the protease peak.

Susceptibility of gram-positive bacteria. Several gram-positive cocci, including *S. aureus*, were utilized in bactericidal assays of the Sephadex G-100 fractions (Fig. 5). The gram-positive bacteria were not sensitive to the bactericidal

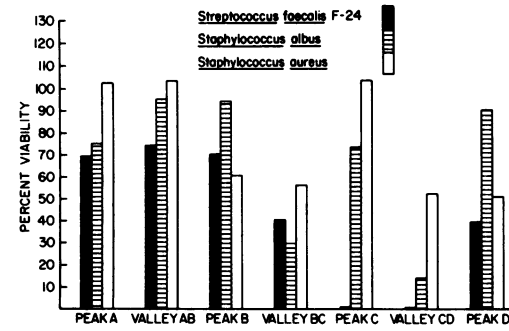

FIG. 5. *Effect of 50 μg of fractionated granule protein on the viability of gram-positive bacteria: S. faecalis F-24, S. albus, and S. aureus.*

activity of valley AB protein, as were the gram-negative bacteria. *S. faecalis* F-24 was killed effectively by the peak C lysozyme and valley CD protein. *S. albus* was sensitive to the valley BC and valley CD regions of the elution profile, but showed 75% viability when tested against the peak C protein. *S. aureus* was not affected by the peak C protein. However, decreases in cell viability were observed in the peak B, valley BC, valley CD, and peak D fractions.

DISCUSSION

The antimicrobial capacities of the PMN have been explained on the basis of two classes of mechanism, those dependent on H_2O_2 and other energy states of oxygen and those independent of them (27). In the present report, we investigated the interactions of human neutrophil granule contents fractionated by Sephadex G-100 gel filtration chromatography with smooth *Enterobacteriaceae* and their rough LPS-deficient mutants. The use of these deep rough mutants has enabled us to localize areas of potent bactericidal activity in the elution profile that appear to be independent of exogenously supplied H_2O_2. These findings are important from two points of view. First, we have localized in an area of low protein concentration a killing capacity that represents a small percentage of the total granule protein. The high specific activity of bactericidal activity against rough mutants in vitro, where achievable concentrations are small, compared with its potential action against smooth bacteria in vivo, where achievable concentrations in phagolysosomes are probably large, may give an indication of the potential significance of the substance(s). Moreover, since the bactericidal activity could not be reproduced by mixing peak A and peak B in various ratios, it appears that the protein species is separate and distinct from any in these major peaks.

Of the bacteria tested, those demonstrating

greater degrees of roughness were most sensitive to the action of the valley AB protein. It has long been established that rough mutants of *S. typhimurium* are less virulent than their smooth parents (1, 2, 11, 13). The information obtained from these studies will allow further investigation to revolve around the isolation and purification of the PMN granule component responsible for the observed killing and an analysis of the role of the mutation in decreasing resistance. The loss of sugar residues in the LPS of rough mutant cell surfaces presumably permits the bacteria to present different substrates to the pool of granule bactericidal and degradative enzymes. A closer analysis of the antimicrobial action of valley AB protein may yield insight into the biochemical mechanism of this decreased resistance to bactericidal activity. Interestingly, the major areas in the Sephadex G-100 elution profile of PMN granule protein that showed bactericidal activity against the gram-positive microorganisms were separate and distinct from the valley AB protein. Gram-positive bacteria were sensitive to areas in the profile that were closely associated with peak C known to contain the bulk of PMN granule lysozyme. This was confirmed by the extreme sensitivity demonstrated by *S. faecalis* F-24, which is known to be lysozyme sensitive. It would appear that lysozyme is the major constituent responsible for the bactericidal activity observed against the gram-positive microorganisms. The fractionation of PMN granule contents by Sephadex G-100 gel filtration chromatography yields two distinct fractions that show independent bactericidal activities against gram-positive and gram-negative bacteria. This presumably indicates fundamental differences in biochemical mechanisms. Whereas viable PMN isolated from patients with chronic granulocytic leukemia have impaired capacities for oxidative killing as compared with normal PMN (27), the bactericidal potential of granule extracts is similar whether obtained from PMN of normal or leukemic donors (21, 22). Further investigation is in progress to identify and define the mechanisms by which these human PMN granule fractions kill bacteria.

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