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Bactericidal Activity of Granule Contents from Rat Polymorphonuclear Leukocytes

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Granule contents from rat polymorphonuclear neutrophils were prepared by extraction with 0.2 M acetate (pH 4), dialyzed against phosphate-buffered saline (pH 7), and tested for bactericidal activity. Bactericidal assays consisted of mixing rat granule extract with 1×10^3 to 3×10^3 bacterial cells per ml at 37°C for 1 h in a medium suited for bacterial growth. The granule extract demonstrated a distinctive dose-dependent bactericidal activity against outer membrane lipopolysaccharide mutants of *Salmonella typhimurium* LT-2, independent of added hydrogen peroxide or other active oxygen derivatives. The rough bacterial mutants showed an ordered increase in sensitivity to the rat lysosomal extracts inversely related to the length of their lipopolysaccharide carbohydrate side chains. Fractionation of the rat polymorphonuclear neutrophil granule extract with Sephadex G-100 column chromatography revealed an elution profile containing three major areas (peaks) of protein. Polyacrylamide gel electrophoresis and examination of enzymatic activity showed that these peaks contained myeloperoxidase (peak A), neutral protease (peak B), and lysozyme (peak C) activities. Also observed in peak C were cationic protein species whose cathodal electrophoretic migration was faster than that for lysozyme. Only peak C exhibited a bactericidal activity against the rough mutants of *S. typhimurium* LT-2 similar to that obtained for the unfractionated granule extract, with susceptibility of the bacterial mutants increasing with a progressive loss of carbohydrate residues in the lipopolysaccharide of the cell wall. The bactericidal activity of the peak C protein fraction was dose dependent. Boiling the unfractionated granule extract or peak C for 30 min had little affect on their antimicrobial activity when reacted against a deep-rough lipopolysaccharide mutant. However, trypsin pretreatment of these fractions significantly reduced their antimicrobial activity for the same mutant chemotype.

Polymorphonuclear neutrophils (PMN) are highly specialized cells responsible for the ingestion, killing, and degradation of invading microorganisms. These cells possess a number of potentially antimicrobial substances and hydrolytic enzymes distributed within their cytoplasmic granules (7, 8, 12, 17, 22, 25, 28). The antimicrobial capacity of PMN granule contents has been the topic of several reviews (6, 22, 25). These mediators of microbicidal activity have been explained on the basis of two types of mechanisms: those dependent on hydrogen peroxide and other energy states of oxygen and those independent of them (6–8, 22, 25). Much of what is known about the antimicrobial activity of PMN granule contents has been derived by adding mixed or fractionated granule components with test microorganisms in in vitro assay systems (12–14, 19–21). The sensitivity of many different gram-positive and gram-negative organisms to specific conditions or fractions has been used to help understand the mechanisms involved, particularly that of the nonoxidative antimicrobial cationic proteins. These studies have described bactericidal components from lysosomes of rabbit, guinea pig, chicken, and human PMN that show substantial selectivity in their antibacterial action against several pathogenic bacteria (1, 2, 4, 15, 17, 30). In the past years, rats have gained considerable importance as an experimental model in the examination of the inflammatory process and in studies involving the humoral or cellular immune responses of humans. Therefore, the characterization of rat PMN granule contents, particularly with regard to bactericidal activity, would aid in future studies that may involve rat leukocytes and possibly contribute to a more thorough understanding of the interrelationships that exist between host defense mechanisms and intrusive bacteria.

In the present report, we have utilized *Salmonella typhimurium* LT-2 and a series of progres-

sively rough lipopolysaccharide (LPS) mutants of this strain to examine the nonoxidative antimicrobial potential of isolated rat neutrophil granule protein. The parent and mutant strains of this organism possess substrates on their surfaces that differ both physically and biochemically and as such can be used as probes for detecting and defining different protein systems involved in the bactericidal process. We have extracted the bactericidal components from rat PMN granules, and the extract was separated into several fractions. The bactericidal activity of the extract and fractions was examined along with a partial physical and biochemical characterization of the granule protein components. Our results are described in this paper. The information obtained from this study will allow further investigation to revolve around the identification, isolation, and purification of the rat PMN granule component(s) responsible for the observed bacterial killing.

MATERIALS AND METHOD

Purification of PMN. The isolation and purification of rat peritoneal PMN was accomplished by the method of Calamai and Spitznagel (3), with minor modifications. Briefly, leukocytes were elicited into the peritoneal cavity of Sprague-Dawley rats (weighing between 250 and 600 g) by intraperitoneal injection of 30 ml of 2.0% glycogen (Sigma Chemical Co., St. Louis, Mo.) in tissue culture medium 199. After 12 h the animals were sacrificed, and 30 ml of tissue culture medium 199 containing 5 U of heparin per ml was injected intraperitoneally. The rat abdomen was massaged, the peritoneum was opened, and the exudate fluid was washed out completely. Pooled exudates were poured through cheesecloth to remove large debris and aggregates. The cells were then pelleted by centrifugation at 126 × *g* for 10 min and suspended to a concentration of 5.0×10^7 cells per ml in tissue culture medium 199 at 4°C. A 4-ml volume of cell suspension was layered onto 3 ml of a 6% Ficoll–10% Hypaque solution (Sigma) and centrifuged at 400 × *g* for 1 h at room temperature. Pelleted cells, consisting of erythrocytes and PMN, were carefully aspirated and pooled. Erythrocytes were removed by hypotonic lysis, and the remaining PMN were washed and suspended in 0.34 M sucrose at 4°C for homogenization. This suspension contained 90 to 99% PMN as determined by Wright-stained smears. Approximately 80 to 100 rats were processed in groups of 25 to 30.

Purification of granules and preparation of granule extracts. The concentration of PMN was adjusted to 4.0×10^8 cells per ml in 0.34 M sucrose. PMN were homogenized with a Teflon pestle and homogenizer until approximately 85% of the cells appeared ruptured when viewed by phase-contrast microscopy. Unbroken cells and nuclei were removed by centrifugation at 126 × *g* for 15 min, and the granules within the supernatant were pelleted by centrifugation at 15,000 × *g* for 30 min. The granule pellet was then suspended in 30 ml of 0.2 M sodium acetate buffer (pH 4.0) supplemented with 10 mM calcium chloride, and the protein was extracted for 24 h with gentle stirring at 4°C. The extraction procedure was repeated with granule debris being removed by centrifugation at 20,000 × *g* for 30 min after each extraction. The supernatant, containing granule extract, was exhaustively dialyzed (2000 molecular weight cutoff membrane; Spectrum Medical Industries, Inc.) against phosphate-buffered saline (pH 7.0) and concentrated by ultrafiltration (Amicon UM-05 filter) to 0.5 to 1.0 mg/ml before use in antimicrobial assays.

Sephadex G-100 column chromatography. Acetate extracts were pooled and concentrated by ultrafiltration (Amicon UM-05 filter) before Sephadex G-100 column chromatography. Approximately 20 to 30 mg of granule protein was chromatographed by previously published procedures (17). After chromatography, fractions were pooled, concentrated by ultrafiltration (Amicon UM-05 filter) to 1 to 3 mg/ml, and dialyzed against phosphate-buffered saline (pH 7.0) before use in bactericidal assays. All operations were conducted at 4°C.

Bacteria and culture conditions. *S. typhimurium* LT-2 and the LPS-deficient mutants derived from it were kindly provided by R. J. Roantree, Stanford University (10). The structures of the LPS of the parent and mutant strains are given in Fig. 1. The organisms were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) to the midlog phase at 37°C on a reciprocal shaker (100 oscillations per min). Growth was followed at 540 nm on a Bausch & Lomb Spectronic 20 spectrophotometer. Crystal violet was used to monitor the degree of roughness of the bacterial strains (24).

Bactericidal assays. Bactericidal assays were done as previously described (19, 20) in a total of 0.4 ml of tryptone-NaCl in 13- by 100-mm culture tubes. Assay mixtures contained 2.0×10^3 to 4.0×10^3 CFU of the appropriate bacteria per ml, and incubations were for 1 h at 37°C. After incubation, 0.1 ml of the assay mixture was plated on Trypticase soy agar, and CFU were counted at 18 to 24 h. Results were expressed as the percentage of viable bacteria compared with controls incubated for 1 h at 37°C, where viable bacteria are defined as those bacteria that can produce colonies on agar. All assay mixtures were done in duplicate with two viable plate counts conducted per assay mixture. Each assay protocol was repeated on at least two separate occasions.

Polyacrylamide gel electrophoresis. Rat leukocyte granule proteins were examined by electrophoresis in acidic polyacrylamide gels by the method of Gabriel (5). Stacking gels of 2.5% acrylamide and 0.625% bisacrylamide were added to separating gels containing 10% acrylamide and 0.15% bisacrylamide. The pH of the beta-alanine gel and running buffer was 4.5. Samples containing 50 μg of granule protein were loaded onto the gels with 6% (final concentration) sucrose and allowed to enter the stacking gel at 2.0 mA per gel for 30 min. The current was increased to 4.0 mA per gel, and electrophoresis was continued until the tracking dye (methyl green) reached the end of the gel. Proteins were stained for one hour with fresh Coomassie brilliant blue G-250 in a methanol-acetic acid-water solution (45:10:45) and destained overnight in the same solution without the protein dye. The migration of standard protein samples of myeloperoxidase (human) and egg white lysozyme (Sigma) were examined in the same manner.

Enzyme and protein determination. Lysozyme activ-

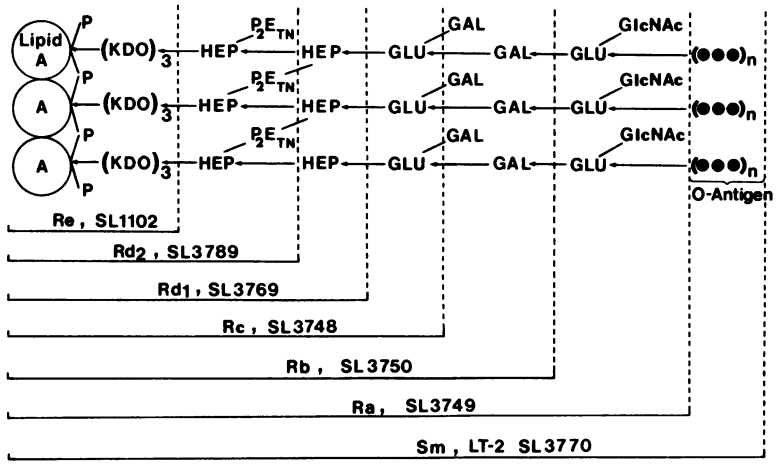

FIG. 1. LPS composition of *S. typhimurium* LT-2 and its rough mutants. Abbreviations: GlcNAc, *N*-acetylglucosamine; GLU, D-glucose; GAL, D-galactose; HEP, L-glycero-D-mannoheptose; KDO, 3-deoxy-D-mannooctulosonic acid; E, ethanolamine; P, phosphate. The structural types of LPS (S through Re) and the strain numbers are indicated. Adapted from McCabe (11).

ity was measured by the lysoplate method of Osserman and Lawlor (18) and by the spectrophotometric method of Shugar (23) with *Micrococcus lysodeikticus* (Sigma) as the substrate and crystalline egg white lysozyme as a standard. Myeloperoxidase was measured by observing the reduction of *o*-dianisidine (Sigma) as described by Worthington Biochemical Corp. (29). The method of Starky and Barret (26) was used to measure proteolytic activity, with azocasein (Sigma) as substrate. Protein was determined by the method of Lowry et al. (9) with crystalline egg white lysozyme as a standard.

Heat denaturation and trypsin pretreatment of granule protein. Samples of rat leukocyte granule protein were pretreated by boiling for 10 and 30 min or with trypsin (20 μg) overnight at 37°C before addition to antimicrobial assays. Appropriate controls containing granule protein incubated at 37°C overnight without trypsin showed no loss of bactericidal activity when tested in antimicrobial assay mixtures. In similar experiments, 25 μg of soybean trypsin inhibitor was added to the trypsin-granule protein mixture before addition to bactericidal assays to ensure that no trypsin activity would contribute to observed antimicrobial activity. Controls involving the addition to the bacterial cells of equal amounts of trypsin or soybean trypsin inhibitor alone did not demonstrate bactericidal activity toward *S. typhimurium* SL1102. The addition of soybean trypsin inhibitor to the granule protein in the absence of trypsin had no observed affect on the antimicrobial activity of the protein samples when mixed with bacterial cells.

Materials and reagents. Trypticase soy broth and agar were from BBL. Tryptone was purchased from Difco Laboratories, Detroit, Mich. TCM 199 was obtained from GIBCO Laboratories, Grand Island, N.Y. Trypsin (TRL-2× crystallized) was from Worthington Diagnostics, Freehold, N.J., and soybean trypsin inhibitor (type II-S) was from Sigma. All other chemicals were of reagent grade.

RESULTS

The elution profile obtained from the Sephadex G-100 column chromatographic separation of acetate-extracted rat PMN granule protein revealed three major peak areas of protein (Fig. 2). Analysis of enzymatic activity (Table 1)

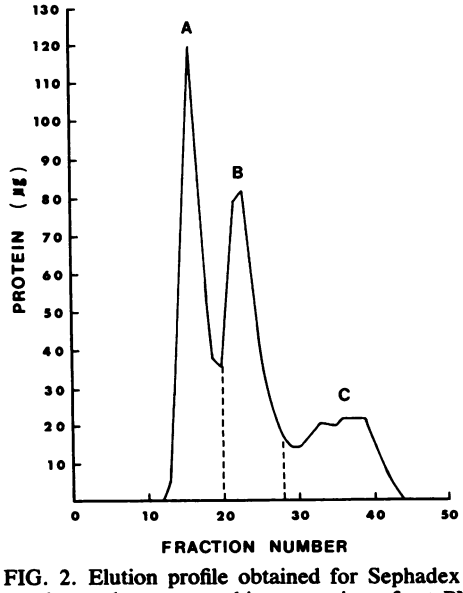

FIG. 2. Elution profile obtained for Sephadex G-100 column chromatographic separation of rat PMN granule protein. Elution from the column (1.5 by 65 cm) was with 0.2 M sodium acetate buffer (pH 4.0) at a flow rate of 12 ml/h. Fractions 1 through 12 represent the void volume. Fraction volumes were 2.0 ml. Dotted lines indicate pooling of fractions.

TABLE 1. Enzyme activities in acetate extracts and fractions from Sephadex G-100 column chromatography

Enzyme	Granule extracts	U/mg of protein		
		Peak A	Peak B	Peak C
Myeloperoxidase[a]	16.9	37.3	1.5	0.09
Neutral protease[b]	0.52	0	1.5	0
Lysozyme[c]	15,294	0	1,053	61,578

[a] One unit of peroxidase activity is that amount of enzyme decomposing 1 μmol of peroxide per min at 25°C. Activity was expressed as the change in optical density at 460 nm per minute per milligram of protein as determined by measuring the reduction of o-dianisidine (29).

[b] One unit of proteolytic activity is that amount of enzyme producing a change in optical density at 366 nm of 1.0 in 30 min at 50°C with azocasein as the substrate (26).

[c] One unit of lysozyme is that amount of enzyme producing a change in optical density at 450 nm of 0.001/ml per min at pH 6.24 at 25°C, with a suspension of *M. lysodeikticus* as the substrate, in a 2.6-ml reaction mixture (23).

showed these peaks to contain myeloperoxidase (peak A), neutral protease (peak B), and lysozyme (peak C). The unfractionated granule extract was resolved into a number of components by cationic polyacrylamide gel electrophoresis. Two components were detected with electrophoretic mobilities against the cathode higher than that for lysozyme, indicating that these proteins are highly cationic. Figure 3 shows the electrophoresis patterns on polyacrylamide gels of the unfractionated rat granule extract, protein peaks obtained by gel chromatography, and of standard proteins of myeloperoxidase and lysozyme. One component (peak A) of the rat granule extract corresponds to the mobility of the myeloperoxidase standard, and another component (peak C) corresponds to the mobility of lysozyme. Also observed in peak C were the two protein species whose cathodal electrophoretic mobility was faster than that for lysozyme (Fig. 3). The unfractionated granule extract and the three major protein areas were concentrated and tested for bactericidal activity.

Bactericidal activity of unfractionated granule extract toward LPS mutants. *S. typhimurium* LT-2 and a series of progressively rough outer membrane LPS mutants derived from it were incubated with various quantities of unfractionated rat granule extract for 1 h at 37°C (Fig. 4). The less carbohydrate that the bacteria possessed in their LPS, the more susceptible they were to the bactericidal activity of even the lowest concentrations of the extracts tested. Of the bacteria tested, those demonstrating greater degrees of roughness (Rc, Rd_1, Rd_2, Re) were most sensitive to the action of the granule extract. The LT-2 smooth parent and the Ra and Rb mutant chemotypes were much less sensitive to the rat granule extract, if affected at all, even at the relatively high concentrations of granule extract tested. Thus, a continuous increase in susceptibility to the bactericidal activity of the granule extract was observed with an apparent transition from a bacteriostatic or slightly bactericidal response (Sm through Rb) to a much greater bactericidal response (Rc through Re) as *S. typhimurium* lost its core polysaccharides.

Susceptibility of LPS mutants to fractionated granule protein. *S. typhimurium* LT-2 and its outer membrane LPS mutants were tested against 50 μg of each Sephadex G-100 pooled peak fraction. Given the assay procedures employed, peak A (myeloperoxidase) and peak B (neutral proteases) demonstrated very little bactericidal activity against any of the mutants (Table 2), with the exception of a significant bactericidal response for peak B against the deep rough Re chemotype. The Re-type mutant SL1102 and the Rd_2-type mutant SL3789 were highly sensitive to the bactericidal activity of the peak C protein fraction. However, there was an increase in antimicrobial resistance of the rough mutants to 50 μg of peak C protein as the degree of smoothness increased from the chemotype Rd_1 to the smooth parent LT-2 strain.

The extreme sensitivity of the deep rough mutants (Rd_2, Re) to the peak C protein fraction

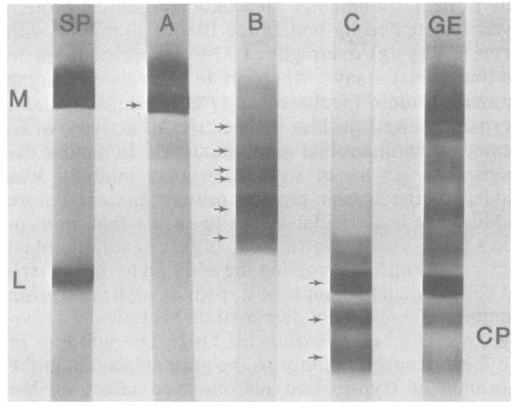

FIG. 3. Cationic polyacrylamide gel electrophoresis of 50 μg each of peaks A, B, and C from the Sephadex G-100 column chromatographic separation of rat PMN granule extract. Also shown is the electrophoretic patterns of 200 μg of the unfractionated granule extract (GE) and of 50 μg of the standard proteins (SP) of myeloperoxidase and lysozyme. Abbreviations: M, myeloperoxidase; L, lysozyme; CP, cationic proteins.

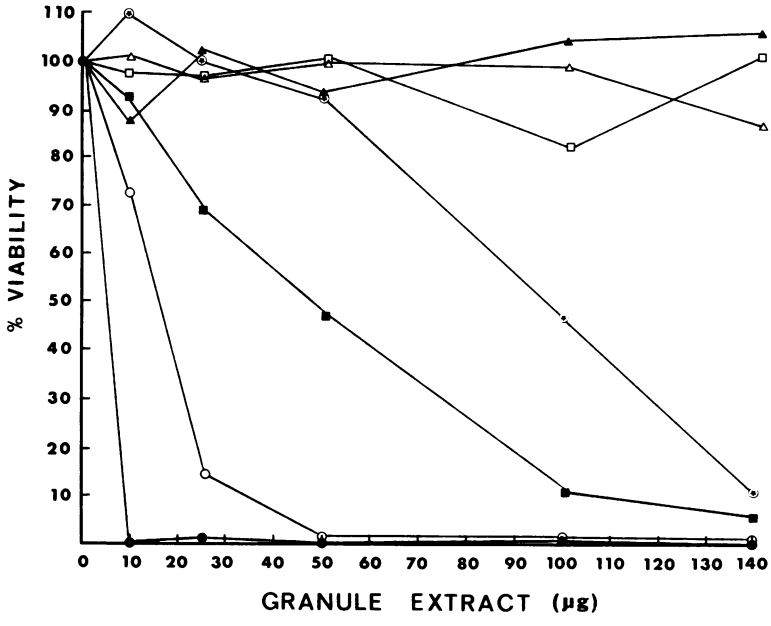

FIG. 4. Bactericidal activity of increasing amounts of rat leukocyte granule extract against *S. typhimurium* LT-2 and its LPS mutants (Ra through Re). Symbols: SL3770, S (△); SL3749, Ra (□); SL3750, Rb (▲); SL3748, Rc (⊛); SL3769, Rd_1 (■); SL3789, Rd_2 (○); SL1102, Re (●). The values shown represent the means of two or more replicate experiments. Standard errors of the means were less than or equal to ±5% viability for all experiments conducted.

(50 μg) prompted an examination of the sensitivity of the mutants of intermediate degrees of roughness to various concentrations of peak C. The sensitivity of the different LPS mutants to the peak C protein fraction was dose dependent. In a manner similar to that observed for the unfractionated granule extract, the various LPS mutants have an ordered increase in sensitivity to lower concentrations of the peak C fraction inversely related to the length of their LPS carbohydrate side chains (Fig. 5).

Thermal denaturation and proteolytic digestion of rat granule protein. Bactericidal activity of 100 μg of unfractionated rat granule extract and 50 μg of peak C fraction for *S. typhimurium* SL1102 (Re chemotype) was only slightly affected after heating in a boiling water bath for 10 min (Table 3). Boiling the fractions for an additional 20 min did not substantially decrease the bactericidal potential. When these fractions were pretreated with trypsin, the antimicrobial activity of the fractions was decreased substantially, indicating a protein nature of the unfractionated granule extract and of the peak C fraction (Table 4). Controls using heat inactivated trypsin (autoclaved) had no affect on the antimicrobial activity of the granule protein tested. Trypsin digests of the protein fractions were monitored by cationic polyacrylamide gel electrophoresis, with substantial digestion of the proteins.

TABLE 2. Effect of 50 μg of pooled peak fractions on the viability of *S. typhimurium* LT-2 and its LPS mutants

Mutant chemotype	% Viability[a]		
	Peak A	Peak B	Peak C
Sm	103 ± 1.3	102 ± 5.8	87 ± 0.5
Ra	100 ± 0.2	86 ± 0.9	104 ± 5.9
Rb	93 ± 1.3	118 ± 1.6	85 ± 1.3
Rc	101 ± 7.2	99 ± 1.6	54 ± 6.3
Rd_1	109 ± 9.3	97 ± 7.8	46 ± 7.0
Rd_2	110 ± 2.5	100 ± 3.7	0 ± 0.0
Re	73 ± 8.6	18 ± 1.5	0 ± 0.0

[a] Data are expressed as the means ± standard errors of two replicate experiments.

DISCUSSION

The mechanisms by which phagocytic cells kill can be classified according to whether or not molecular oxygen or its reduction products are participants in the process, thereby designating these mechanisms as either oxygen dependent or oxygen independent (7, 8, 22, 25). A well-known oxygen-dependent system consists of myeloperoxidase, active O_2 derivatives such as H_2O_2, O_2^- and OH·, a low concentration of halide ion, and either iodide or chloride and is dependent upon PMN metabolism (22). In addi-

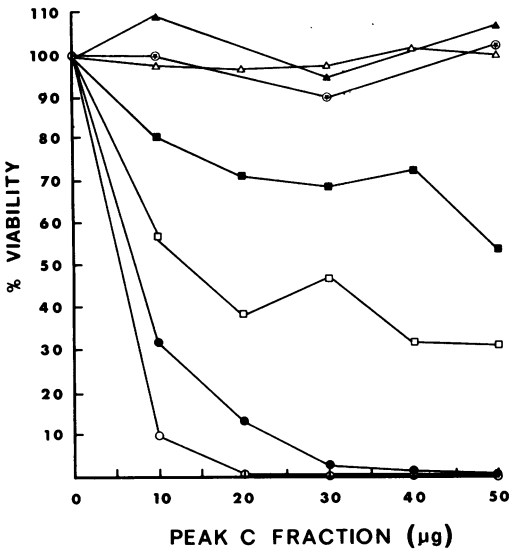

FIG. 5. Bactericidal activity of increasing amounts of rat leukocyte peak C protein against *S. typhimurium* LT-2 and its LPS mutants (Ra through Re). Symbols: SL3770, S (⊛); SL3749, Ra (▲); SL3750, Rb (△); SL3748, Rc (■); SL3769, Rd_1 (□); SL3789, Rd_2 (●); SL1102, Re (○). The values shown represent the means of two or more replicate experiments. Standard errors of the means were less than or equal to ±5% viability for all experiments conducted.

tion, numerous nonoxidative mediators of PMN microbicidal activity, with varied degrees of importance, have also been described. Among these are shifts in intraphagolysosomal pH, lysozyme, lactoferrin, cationic proteins, and neutral proteases (22). Of these mediators, the cationic proteins have been widely implicated as the primary nonoxidative antimicrobial factor. These low-molecular-weight, highly cationic proteins have been observed in guinea pigs, rabbits, and humans (16, 17, 30) and demonstrate a distinct antimicrobial activity for a number of different bacterial species. The killing of gram-negative organisms by these proteins appears to vary depending on the structure of the bacterial outer membrane. LPS mutants are killed quite readily by the action of these low-molecular-weight cationic proteins, whereas smooth variants are more resistant (13, 14, 18, 19). Recently, Weiss et al. (28) have isolated a high-molecular-weight cationic protein from human PMN which increases the envelope permeability of susceptible gram-negative bacteria and disrupts the synthesis of macromolecules in these organisms.

In the present study, we have utilized the differential sensitivity of rough LPS mutants of *S. typhimurium* LT-2 as target bacteria in in vitro assay mixtures to examine the antibacterial activity of isolated granule components derived from rat PMN. The use of these mutants has enabled us to localize areas of potent bactericidal activity from fractionated rat granule protein that appear to be independent of added hydrogen peroxide or other active oxygen derivatives. In doing so, we have initiated a characterization of the rat PMN granule components, particularly with regard to their bactericidal activity. Data presented reveals that acetate-extracted granule contents from rat PMN possess a bactericidal activity against outer membrane LPS mutants of *S. typhimurium* LT-2. Those mutants demonstrating greater degrees of roughness were most sensitive to the granule extract. Smooth variants seemed to be only growth inhibited by even high concentrations of granule extracts, whereas mutants with a more substantial loss of core LPS were killed by the same extracts at lower concentrations.

Rat PMN granule extracts separated by Sephadex G-100 column chromatography resolved into three major peaks. Cationic polyacrylamide gel electrophoresis and analysis of enzyme activity revealed these peaks to contain myeloperoxidase, neutral protease, and lysozyme activities (peaks A, B, and C, respectively). Two components were detected cathodal to the lyso-

TABLE 3. Effect of boiling on the bactericidal activity of unfractionated granule extract (100 μg) and of the peak C fraction (50 μg) against *S. typhimurium* SL1102 (Re)

Rat granule protein	Treatment	% Viability[a]
Unfractionated granule extract	No treatment	0
	Boiled (10 min)	15 ± 3.8
	Boiled (30 min)	23 ± 4.2
Peak C fraction	No treatment	0
	Boiled (10 min)	4 ± 0.0
	Boiled (30 min)	12 ± 2.1

[a] Data are expressed as means ± standard errors for two replicate experiments.

TABLE 4. Effect of trypsin on the bactericidal activity of unfractionated granule extract and of the peak C fraction against *S. typhimurium* SL1102 (Re)

Rat granule protein	Treatment	% Viability[a]
Unfractionated granule extract (100 μg)	No treatment	1 ± 0.3
	Trypsin (20 μg)	96 ± 5.6
Peak C fraction (20 μg)	No treatment	4 ± 2.0
	Trypsin (20 μg)	85 ± 14.8

[a] Data are expressed as means ± standard errors for two replicate experiments.

zyme band, suggesting that these proteins are highly cationic. Our partial physical characterization of the rat PMN granule contents is in agreement with a previous report (E. G. Calamai and J. K. Spitznagel, Fed. Proc. 36:1189, 1977), the details of which have yet to be published. Upon examination of the various peaks for bactericidal activity, we found only peak C (lysozyme plus cationic proteins) to be effective in killing the outer membrane mutants of *S. typhimurium* LT-2 with the assay design employed. Similar to the unfractionated granule extract, the antibacterial activity of peak C was dose dependent.

Both the unfractionated rat granule extract and the peak C fraction maintained almost all of their bactericidal potential even after boiling for 30 min. The findings of Walton (27) and Odeberg and Olsson (16) have well established the existence of heat-stable cationic proteins from both rabbit and human PMN. These proteins retained their bactericidal activity after heating and appear to function due to their cationic properties rather than their enzymatic activities. That this is true for the isolated bactericidal proteins from rat PMN granules is also indicated at this time. Pretreatment of the granule extract or peak C with trypsin substantially abolished their antimicrobial activity, suggesting that the bactericidal component(s) is of a protein nature.

More information about the antimicrobial activity of isolated rat PMN granule proteins will be gained from studies in progress involving further separation, identification, and purification of the antimicrobial component(s).

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