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Susceptibility of Lipopolysaccharide Mutants to the Bactericidal Action of Human Neutrophil Lysosomal Fractions

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Acetate extracts of purified human neutrophil granules (a mixed population containing specific and azurophil granules) were dialyzed against phosphate-buffered saline (pH 7.0) and tested for bactericidal activity against smooth parent and rough mutant, gram-negative bacteria. Rough (Re) mutants of *Escherichia coli*, *Salmonella typhimurium*, and *Salmonella minnesota* were exquisitely more sensitive to extracts of human polymorphonuclear leukocyte granules than were their smooth (S) parents. The mean lethal dose (LD_{50}) for the parent strains was 25 to 50 μg of granule protein per ml. As much as 500 μg of extract per ml failed to kill 100% of the S parents. The LD_{50} for the rough mutants was 1.5 to 2.0 μg of the same granule extract per ml; 100% killing occurred with 5 to 10 μg of lysosomal protein per ml. Conditions affecting the growth of the bacteria greatly affected their sensitivity to the granule extracts. Granule extract killed bacteria grown with aeration to log phase 10 to 15 times more efficiently than the same bacteria grown to stationary phase under static conditions. The bactericidal incubation mixture also influenced results, in that greater killing occurred with tryptone than with phosphate or *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid-buffered saline. Bactericidal activity depended on lysosomal protein concentration, time, and temperature. Boiled lysosomal fractions failed to kill the S parents but retained 20 to 50% of their ability to kill the Re mutants. Parents (smooth) were killed more efficiently at pH 5 to 6, whereas their Re mutants were killed more efficiently at pH 7 to 8.

There have been many approaches to the problem of defining the mechanisms of the bactericidal and degradative action of human neutrophils. Investigators have observed the bactericidal and degradative properties of neutrophil extracts (9, 17), neutrophil granule extracts (19, 39), and purified granule proteins such as myeloperoxidase (15, 22), lactoferrin (24, 30), elastase (13, 14), collagenase (29), cationic proteins (29), lysozyme (34, 38), and proteinases (39). Such studies help define specific bactericidal mechanisms in vitro. Experiments performed with human neutrophils that possess intrinsic dysfunctions, such as defective oxidative metabolism (11, 12) or lack of myeloperoxidase (19), have shown that several competent bactericidal systems operate within neutrophils. Experiments with animal neutrophilic leukocytes have shown that some animals naturally lack certain lysosomal enzymes, (chickens lack MPO [5] and cattle lack lysozyme [31]), or have oxidative metabolism (32) or intraphagosomal pH (23, 37) that differs from human neutrophils. These differences have been useful in comparative studies, but, because of these differences, conclusions drawn from work with animal lysosomal contents cannot be directly equated with the results of experiments with human material.

In the present report, we investigate the interactions of normal human neutrophil granule fractions with smooth *Enterobacteriaceae* and with their lipopolysaccharide (LPS)-deficient mutants. The parents and mutants possess different substrates on their surfaces which they present to the pool of granule bactericidal and degradative enzymes. The mutants can be used then as probes for different enzyme systems involved in the bactericidal process. Bacterial LPS mutants have been used effectively to study virulence in animal models (23, 25) and to investigate the bactericidal action of neutrophil lysosomal fractions from guinea pigs (7, 8). Such studies showed that rough mutants of *Escherichia coli* and *Salmonella typhimurium*, lacking some or all of their O antigen and core polysaccharides, were more susceptible to phagocytosis (25) and more sensitive to guinea

pig neutrophil granule contents (7, 8) and human serum bactericidal agents (27) than were their smooth parents.

MATERIALS AND METHODS

Neutrophil purification. Neutrophils were obtained from heparinized (5 to 10 U/ml) venous blood of healthy donors of both sexes, by dextran or plasmagel sedimentation, centrifugation over Ficoll-Hypaque, and hypotonic lysis as described previously (32, 36). Cell types were determined by observation of Wright-stained cytocentrifuge slides. Neutrophil suspensions contained 90 to 95% neutrophils, 3 to 8% eosinophils, 0 to 2% monocytes, and 0 to 2% lymphocytes. Platelets and erythrocytes were not detectable. One unit of blood yielded 1.0×10^9 to 1.2×10^9 polymorphonuclear leukocytes (PMNs).

Granule extracts. Neutrophils, suspended in 0.34 M sucrose (3×10^8/ml), were homogenized to 85 to 90% breakage in a Teflon glass homogenizer and centrifuged at $126 \times g$ for 15 min as described previously (36; R. F. Rest and J. K. Spitznagel, Biochem. J., in press). The $126 \times g$ supernatant was centrifuged at $20,000 \times g$ for 20 min to yield a pellet containing a mixed population of granules (two classes of azurophil granules and one class of specific granules). The $20,000 \times g$ pellet was extracted overnight at 3°C with 4 ml of 0.2 M acetate buffer (pH 4.0) containing 0.01 M $CaCl_2$. The extract was centrifuged at $20,000 \times g$ for 20 min and the pellet was reextracted overnight. The two supernatants were combined and dialyzed free of acetate against phosphate-buffered saline (pH 7.0) (PBS) in an Amicon MMC concentrator over a UM-2 membrane. This dialyzed extract was clarified by centrifugation at $20,000 \times g$ for 20 min, and will be called the granule extract. Granules from 1.2×10^9 neutrophils yielded about 10 mg of extract protein.

Bacteria. The smooth (S) gram-negative bacteria used in these experiments possessed a complete LPS molecule with O antigen and core; their deep rough (Re) mutants possessed no O antigen and lacked all core polysaccharides including the heptoses. They did contain 2-keto-3-deoxyoctulosonic acid (KDO). *E. coli* O111:B4 and its Re mutant RC59 were obtained from Loretta Leive of the National Institutes of Health (16, 26). *S. typhimurium* SL1027 and its Re mutant SL1102 and *Salmonella minnesota* 1114 and its Re mutant R595 were gifts of Alois Nowotny of Temple University (28). Bacteria were grown to stationary phase overnight in Trypticase soy broth with or without aeration. To obtain log-phase cells, cultures aerated overnight were transferred (1:100) to 40 ml of warm Trypticase soy broth in 250-ml Erlenmeyer flasks and shaken in a 37°C reciprocal-shaker water bath. Cells were harvested when cultures achieved an optical density at 650 nm (OD_{650}) of 0.50, equivalent to 5×10^8 cells/ml, washed once in cold saline or PBS, diluted in 0.5% tryptone containing 0.5% NaCl (tryptone-NaCl), and kept on ice until used.

Bactericidal assays. Bactericidal assays were done in a total of 0.2 ml of tryptone-NaCl (pH 7.0) in the wells of a small plastic tray. The assay mixtures contained 2×10^3 to 4×10^3 colony-forming units (CFU) 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 (containing 0 to 400 CFU) was plated on Trypticase soy agar plates and CFU were counted at 18 to 24 h. Results are expressed as percentage of viable bacteria, after the appropriate incubation, where viable bacteria are defined as those bacteria that can produce colonies on agar. No clumping of bacteria was observed in Gram-stained smears of the incubation mixtures.

Protein. Protein was measured by the method of Lowry et al. (20) with chicken egg white lysozyme as standard.

Materials. Trypticase soy broth and agar were from BBL. Tryptone was purchased from Difco. Plasmagel was obtained from HTI Corp., Buffalo, N.Y. All other chemicals used were of reagent grade.

RESULTS

Effect of growth and incubation conditions on the susceptibility of bacteria to granule extracts. We tested various conditions of optimal killing in the bactericidal assays, and at the same time attempted to approximate putative intraphagosomal conditions. Granule extracts killed aerobically grown, log-phase bacteria (both the S parents and Re mutants) to a greater extent and more quickly than they killed statically grown, stationary cells (Table 1). The granule extract also killed all the bacteria tested to a greater extent when a nutrient broth (tryptone) was used as the incubation medium as opposed to when a non-nutrient source such as PBS or *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline (pH 7.0) was used (Table 2). With these observations in mind, assays were done subsequently with aerated, log-phase bacteria in tryptone-NaCl.

TABLE 1. *Susceptibility to granule extract of statically grown, stationary phase versus aerated, log-phase bacteria*[a]

Strain	Viable bacteria (%)	
	Static, stationary phase	Aerated, log phase
Escherichia coli O111:B4	78	86
E. coli RC59	5	0
Salmonella minnesota 1114	97	51
S. minnesota R595	6	0
Salmonella typhimurium SL1027	74	44
S. typhimurium SL1102	4	0

[a] Fifty micrograms of protein per ml.

Susceptibility of S versus Re bacteria. The three different pairs (parent-mutant) of gram-negative bacteria possessing S or Re LPS were incubated with varying quantities of granule extract for 1 h (Fig. 1) or with a single dose (75 μg/ml) of granule extract over time (Fig. 2). The Re mutants of all three species tested were much more sensitive to the bactericidal action of granule extracts than were the S parents. Throughout the experiments both the S and Re *E. coli* seemed to be killed less effectively by the extracts than were the respective *Salmonella*. The mean lethal dose (LD_{50}) of the extracts of all three Re mutants was 3 to ≥30 times less than the LD_{50} for their S parents. The LD_{50} for the Re mutants was 5 to 10 μg/ml, and for the S parents it was 25 to 250 μg/ml. Re mutants were killed ≥99% by about 35 μg of protein per ml whereas the S parents were never killed more than 70% by as much as 500 μg of protein per ml. Killing of the Re mutants was complete in 1 h or less, with a higher rate of killing in the first half of incubation than in the last half.

TABLE 2. *Bactericidal activity of granule extract*[a]

Bacteria	Viable bacteria (%)		
	Tryptone-NaCl	PBS	HEPES
Escherichia coli O111:B4	86	118	124
E. coli RC59	0	6	142
Salmonella minnesota 1114	51	67	102
S. minnesota R595	0	3	4
Salmonella typhimurium SL1027	44	55	54
S. typhimurium SL1102	0	10	1

[a] Fifty micrograms of protein per ml in 0.5% tryptone-0.5% NaCl, PBS, or 0.1 M HEPES buffer, all at pH 7.0. Aerated, log-phase cells.

Effect of bacterial concentration. The assays described in this paper were performed with bacteria at a concentration of 2×10^3 to 4×10^3 CFU/ml to simplify and expedite dilution and plating of bacteria at the completion of the assays. To assure ourselves that the assays were being performed well below the limits of bactericidal capacity of the extracts, a constant amount of extract was tested with increasing concentrations of bacteria (CFU/ml). The results (Fig. 3) show that the amount of killing by granule extracts was essentially constant, with initial bacterial concentrations ranging from 2×10^3 to 3×10^3 CFU/ml to 2×10^7 to 3×10^7 CFU/ml.

Effect of incubation pH. The intraphagolysosomal pH of human neutrophils slowly de-

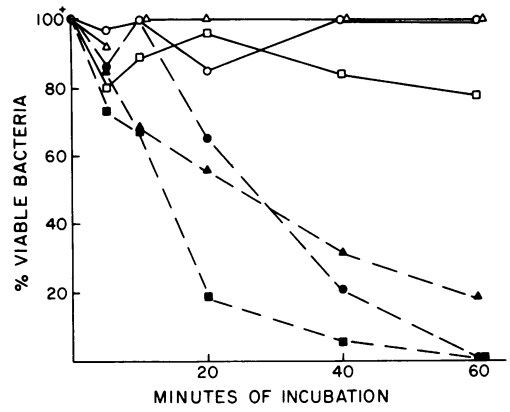

FIG. 2. *Bactericidal activity of granule extract over time (75 μg of protein per ml). Symbols: ○, E. coli O111:B4; □, S. minnesota 1114; △, S. typhimurium SL1027; ●, E. coli RC59; ■, S. minnesota R595; ▲, S. typhimurium SL1102.*

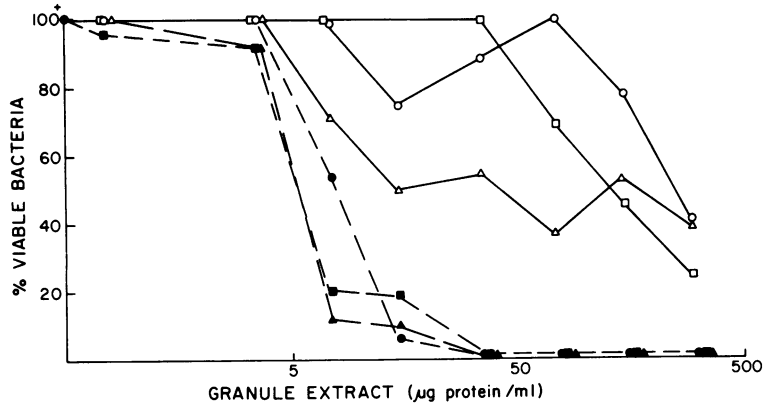

FIG. 1. *Bactericidal activity of increasing amounts of granule extract. Symbols: ○, E. coli O111:B4; □, S. minnesota 1114; △, S. typhimurium SL1027; ●, E. coli RC59; ■, S. minnesota R595; ▲, S. typhimurium SL1102.*

creases from near neutrality to about pH 6, 1 h after phagocytosis (23). The many enzymes found within human neutrophil granules have varying pH optimums ranging from the neutral proteases with optima near pH 7 (29) to the acid glycosidases and proteinases (2, 39), and we postulated that the Re mutants presented different surface substrates to the granule enzymes. Bactericidal assays were therefore performed with granule extracts over a pH range of 5 to 8, using an incubation mixture containing tryptone-NaCl, buffered with 0.05 M potassium phosphate (Fig. 4). Bacterial viability in

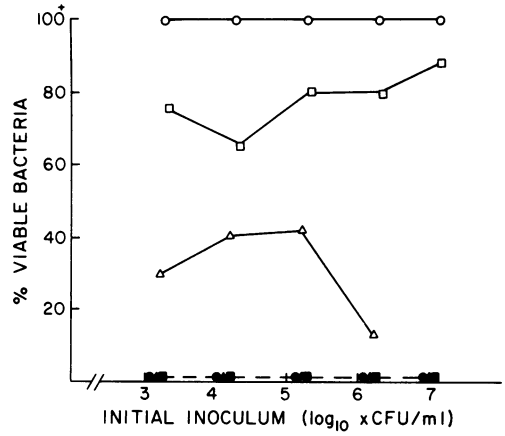

FIG. 3. *Bactericidal activity of granule extract on increasing concentrations of bacteria (100 μg of protein per ml). Symbols: ○, E. coli 0111:B4; □, S. minnesota 1114; △, S. typhimurium SL1027; ●, E. coli RC59; ■, S. minnesota R595; ▲, S. typhimurium SL1102.*

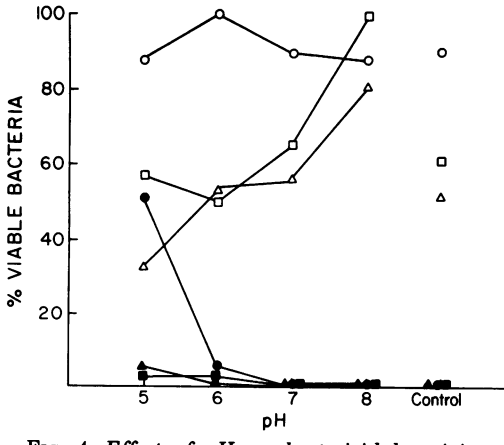

FIG. 4. *Effect of pH on bactericidal activity of granule extracts (50 μg of protein per ml). Results at the different pH values are compared with controls run at the respective pH values. Symbols: ○, E. coli O111:B4; □, S. minnesota 1114; △, S. typhimurium SL1027; ●, E. coli RC59; ■, S. minnesota R595; ▲, S. typhimurium SL1102.*

controls was not affected by the range of pH values studied. When incubated with granule extract, the S parents were killed to a greater extent at pH 5 and 6, whereas the Re mutants were killed to a greater extent at pH 7 and 8.

Effect of incubation temperature. To understand the possible involvement of enzymes in the killing by granule extracts and to see if there were any components in the extracts that perhaps were tightly bound to the bacteria, bacteria plus granule extract were incubated at different temperatures (3, 22, and 37°C) for 1 h and then plated for viability at 37°C. Except for one of the Re mutants (*S. typhimurium* SL1102), no killing was observed with the bacteria incubated at 3°C, with intermediate killing observed at 22°C (Table 3). With all parents and mutants, killing was greatest at 37°C.

Bactericidal activity of boiled granule extracts. To further distinguish between enzymatic and non-enzymatic bactericidal activity, mixed granule extracts were boiled for 10 or 30 min and the bactericidal activity was tested (Table 4). The bactericidal activity of these

TABLE 3. *Effect of incubation temperature on the bactericidal activity of granule extract*[a]

Strain	Viable bacteria (%) at:		
	3°C	22°C	37°C
Escherichia coli O111:B4	100	86	54
E. coli RC59	92	44	0
Salmonella minnesota 1114	129	91	71
S. minnesota R595	105	71	1
Salmonella typhimurium SL1027	112	74	49
S. typhimurium SL1102	79	55	10

[a] One hundred fifty micrograms of protein per ml. Results at the different temperatures are computed based on controls run at the respective temperatures.

TABLE 4. *Bactericidal activity of boiled granule extract*[a]

Strain	Viable bacteria (%)		
	Granule extract	Granule extract boiled for 10 min	Granule extract boiled for 30 min
Escherichia coli O111:B4	76	90	131
E. coli RC59	2	79	93
Salmonella minnesota 1114	54	132	123
S. minnesota R595	0	36	41
Salmonella typhimurium SL1027	36	112	98
S. typhimurium SL1102	2	54	74

[a] One hundred fifty micrograms of protein per ml.

boiled extracts towards the S parents was completely abrogated. The Re mutants of *E. coli*, *S. typhimurium*, and *S. minnesota* were killed 7, 50, and 26%, respectively, by extracts that had been boiled for 30 min.

DISCUSSION

Our results confirm and extend the concept that outer membrane mutants of the *Enterobacteriaceae* provide useful probes of the molecular and biochemical basis of the bactericidal activity of human neutrophil granule fractions. The outer membrane of gram-negative bacteria is important in determining initial contact with and subsequent phagocytosis by neutrophils, and is essential in determining the microbe's susceptibility to the bactericidal factors of the leukocyte. Rough (Re) mutants, lacking all sugar residues in the LPS except for KDO, were at least 10 to 40 times more sensitive to the bactericidal activity of PBS-dialyzed acetate extracts of human neutrophil granules than were their smooth (S) parents. Using a series of LPS mutants, possessing diminishing quantities of sugars in their LPS (Ra through Re mutants), Friedberg and Shilo showed that a complete LPS core was essential for resistance of *S. typhimurium* to the bactericidal effects of guinea pig neutrophil lysosomal extracts (7, 8). It is reasonable to extrapolate results of others and the results of our own investigations to the conclusion that gram-negative bacteria are resistant to the bactericidal action of lysosomal contents due to the carbohydrate in their LPS. The mechanisms which underlie this action of LPS remain to be rigorously studied and proven. The extreme sensitivity of Re mutants to extracts of human neutrophil granules may be due to more than just the mutants' lack of O antigen, core polysaccharides, and heptose, and thus the absence of a simple steric barrier. Studies on the biochemical and physical structure of the outer membrane (OM) of Re mutants of *S. typhimurium* and *E. coli* have shown the following: rough OM has a higher phospholipid to protein ratio (two- to fourfold) than smooth OM due mostly to a loss in OM proteins; rough OM is "split" during freeze fracture whereas smooth OM is not; and the glycolipids of the S and Re mutants are probably very similar (1, 3, 35).

In our studies the bactericidal activity of granule extracts was expressed to a greater degree when bacteria and extract were incubated together in a growth medium, rather than in a nongrowth medium such as buffer. These results indicate that the physiology and metabolism of the bacteria are important factors in their susceptibility to killing by neutrophils. This is important because pathogens in vivo are probably in a growth environment, be it in blood, tissue, or within the host phagocytic cells. In vitro studies of intraleukocytic killing are usually performed in growth medium primarily to keep the leukocytes viable; in these instances the bacteria too are in growth conditions. Most reported in vitro bactericidal studies using human neutrophil fractions have been done with buffers, most often phosphate, acetate, or citrate, often at pH values not observed in vivo (9). Many of these are by themselves toxic to the bacteria that are being studied (Spitznagel, personal communication). Few investigators take the physiology and metabolism of the bacteria into account in the study of the bactericidal activity of leukocyte material. An exception to this is the work of Elsbach, concerning the effects of rabbit granulocyte fractions on the physiology, metabolism, and viability of *E. coli* (4, 6). Using an incubation mixture containing Casamino Acids at pH 7.4, he reported that, although *E. coli* lose their ability to divide almost immediately after contact with lysosomal fractions, the bacteria retain their ability to continue protein synthesis.

We also observed an increased susceptibility of aerated, log-phase bacteria (versus statically grown, stationary-phase bacteria) to the bactericidal action of human neutrophil granule fractions. Others have observed similar effects with a wide range of substances, including lysosomal fractions of guinea pigs (7, 8). Ginsberg et al. observed this effect with human neutrophil extracts that were obtained by a completely different procedure than that used in our lab (9). Working with fractions of human neutrophils and "enzyme cocktails," they observed increased sensitivity of aerated gram-positive bacteria but failed to observe increased sensitivity of aerated gram-negative organisms. The reasons for this discrepancy are unclear but might be explained by the nonphysiological assay system used by that group, i.e., a nongrowth medium (acetate buffer) at pH 5.

The bactericidal activity of the lysosomal fractions was dependent in our experiments upon time and the concentration of lysosomal protein; and was independent of the ratio of granule extract protein to numbers of bacteria, between 10^3 and 10^7 CFU/ml. Although comparison of these types of data from different laboratories is difficult because of the different extraction and incubation procedures used, the amount of human neutrophil lysosomal extract needed for 50% killing of the S parents (25 to 250 μg/ml) and the Re mutants (5 to 10 μg/ml, equivalent to 1×10^7 to 2×10^7 PMN) appeared

to be less than or equal to the amounts of lysosomal or neutrophil extracts needed for bactericidal activity reported for guinea pigs (7, 8), chickens (5), and rabbits (41).

The observation that the S parents were killed best at acid pH, whereas the Re mutants were killed more effectively at or above pH 7, is of considerable interest. Conceivably, the different substrates present on the smooth and rough bacteria are attacked by enzymes with different pH optima. For instance, since the lysosomal glycosidases have an acid pH optima, they would possibly be needed to degrade the polysaccharide coat of the smoother organisms in order to let other enzymes (perhaps with neutral pH optima) attack a site that was previously hidden.

Odeberg and Olsson showed that the bactericidal activity of purified human cationic proteins towards *E. coli* was heat stable (29), and was therefore due to the cationic properties of the proteins, not their enzymatic activities. Boiling of the granule extracts used in our study completely abolished the bactericidal activity toward the S parents, whereas boiling only partially inhibited the bactericidal activity of the extracts toward the Re mutants. Similarly, the bactericidal activity of the granule extracts was temperature dependent, with little or no killing of either the S parents or Re mutants occurring at 3°C. The lack of killing observed with boiled extract or with normal extract at 3°C showed that the majority of the bactericidal activity under these conditions was not due simply to ionic interactions (e.g., of bacteria with some or all of the cationic proteins) but was due to enzymatic activity.

Killing appeared not to be due to H_2O_2-dependent systems, since (i) no H_2O_2 was added to the system, and (ii) even if H_2O_2 were somehow produced at low levels by the granule extract, the bactericidal activity of H_2O_2/MPO/Cl system is inhibited by the tryptone-NaCl incubation medium used (unpublished observations). Oxidative bactericidal mechanisms involving cytosol enzymes and cofactors, e.g., reduced nicotinamide adenine dinucleotide phosphate (NADPH), glutathione, NADPH oxidase, and glutathione peroxidase, also appeared not to be involved in the bactericidal activity since these enzymes and cofactors are separated from the granules in the preparative scheme. These possibilities are being investigated at the present time.

It was reassuring to observe that all three parent-mutant pairs reacted similarly to the various experimental manipulations used in this study. The sensitivity of the rough bacteria can thus be taken tentatively as a general phenomenon, without having to question the role of minor or yet-undefined genetic differences between a specific S parent and its Re mutant that do not specifically involve the outer membrane.

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