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Bactericidal Activity of Specific and Azurophil Granules from Human Neutrophils: Studies with Outer-Membrane Mutants of *Salmonella typhimurium* LT-2

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Extracts of specific granules and azurophil granules from human neutrophils were tested for their bactericidal activity against various lipopolysaccharide mutants of *Salmonella typhimurium* LT-2. Three purified granule populations, one specific and two azurophil, were obtained by isopycnic centrifugation of homogenized neutrophils. Each was extracted with 0.2 M acetate buffer (pH 4), and the extracts were dialyzed against phosphate-buffered saline (pH 7) to remove acetate. These extracts contained ≥84% of the lysozyme, lactoferrin, or myeloperoxidase initially present in the whole granules. The *S. typhimurium* mutants possessed Ra, Rc, Rd_1, Rd_2, or Re lipopolysaccharide. As the carbohydrate content of the lipopolysaccharide decreased, the bacteria became increasingly more susceptible to the bactericidal activity of all granule extracts. Bactericidal activity of the extracts was in the order: mixed (azurophil + specific) ≥ azurophil ≫ specific. Specific granules were bacteriostatic for S through Rd_2 bacteria. They were bactericidal only for the Re mutant. Both azurophil granule populations were equally bactericidal. Extracts boiled for 30 min retained none of their bactericidal activity for any of the bacteria; however, they remained bacteriostatic for the deep rough (Rd_2, Re) mutants. Bactericidal activity was dependent upon pH, in that mixed and azurophil granule contents killed the smooth parent and Ra mutant best at pH 5, the Rc and Rd_1 mutants to the same degree at pH 5 to 8, and the deep rough mutants (Rd_2 and Re) best at pH 8. Specific granule contents were most bacteriostatic for S through Rd_2 bacteria at pH 5 and killed the Re mutant only at pH 8. Thus, as the *S. typhimurium* lipopolysaccharide content decreased, the bactericidal pH optimum increased. Killing by all extracts was dependent upon incubation temperature, with almost no bactericidal or bacteriostatic activity observed when bacteria and granule fractions were incubated on ice (2°C) and plated immediately. Intermediate killing was observed at 22°C. If bacteria were incubated with granule extracts at 2°C, washed free of extract, suspended in medium without extract, and reincubated at 37°C, killing was observed. This suggested that a component(s) of the extracts was sticking to the bacteria at 2°C but killing only at 37°C.

The granules (lysosomes) of human neutrophils contain enzymes that can at least partially degrade most molecules found on the surface of gram-negative bacteria. After phagocytosis of bacteria, the granule-associated degradative enzymes and other bactericidal proteins are released into the phagocytic vacuole, which fuses with these granules. At least four physically and biochemically distinct populations of granules exist: (i) a structurally heterogeneous population of specific granules, banding at a single modal density, that contains lactoferrin, lysozyme, and few other identified protein components (6); (ii) and (iii) two populations of azurophil granules, banding at two different modal densities that each contain myeloperoxidase, lysozyme, cationic proteins, and many more acid and neutral hydrolases (including proteases, phospholipases, and glycosidases; 1, 5, 17, 19); and (iv) a population of "tertiary" granules that are elusive and poorly defined (1, 5). Isopycnic centrifugation techniques developed in this laboratory allow us to obtain purified preparations of the different granule populations (14), and in this report we investigate the contribution of each population to neutrophil bactericidal activity.

Neutrophil bactericidal mechanisms can be divided into two groups, those mechanisms dependent upon oxidative processes, e.g., the myeloperoxidase-Cl^--H_2O_2 system and O_2, and those independent of oxidative processes, e.g., lysozyme, cationic proteins, and apolactoferrin. The oxidative bactericidal processes depend largely upon soluble enzymes or cofactors present in the cytosol and enzymes located on or in membranes; the nonoxidative processes appear to depend solely upon proteins found within the neutrophil granules. In vivo, both processes certainly work together.

To investigate the mechanisms of the bactericidal activity of polymorphonuclear neutrophil (PMN) granule fractions, we previously used deep rough outer-membrane mutants of some of the *Enterobacteriaceae* (13). In this report, we used *Salmonella typhimurium* LT-2 and its rough mutants that contain progressively less carbohydrate in their lipopolysaccharide (LPS). These mutants can be seen as presenting to the granule enzymes a gradually changing series of substrates that are different both physically (e.g., hydrophobic versus hydrophilic) and biochemically (e.g., newly exposed carbohydrates, lipids, and proteins). It is possible that these mutants might represent the degradative sequence that occurs in vivo within the phagolysosome shortly after phagocytosis.

MATERIALS AND METHODS

Purification of PMN. Neutrophils were purified from fresh, heparinized (10 U/ml) human blood by dextran and Ficoll-Hypaque sedimentation as described previously (14). Final cell suspensions were at least 92% PMN.

Purification of granule populations. Homogenized PMN (in 25% sucrose [wt/vol]) were separated over linear (30 to 53% [wt/vol]) sucrose density gradients by isopycnic centrifugation to yield one population of specific granules and two populations of azurophil granules as described previously (14). A mixed granule fraction containing both azurophil and specific granules was obtained by differential centrifugation (20,000 × *g* for 20 min) of a postnuclear supernatant (126 × *g* for 15 min) of homogenized PMN as described previously (14).

Preparation of granule extracts. Fractions of the sucrose density gradient that contained the separate granule populations were appropriately pooled, diluted with one part 0.15 M NaCl, and centrifuged at 50,000 × *g* for 30 min to obtain pellets. Granule pellets were suspended in and extracted with 0.2 M sodium acetate buffer (pH 4) containing 0.01 M $CaCl_2$, as previously described (13, 14). Extracts were dialyzed free of acetate against phosphate-buffered saline (pH 7, containing 7.4 g of NaCl, 0.285 g of KCl, 0.29 mg of $Na_2HPO_4 \cdot 7H_2O$, and 0.083 g of KH_2PO_4 per liter of distilled, deionized water) in an MMC concentrator/dialyzer (Amicon Corp., Lexington, Mass.) over a UM-2 membrane (molecular-weight cut off, 1,000) or in dialysis tubing (molecular-weight cut off, 3,500).

Bacteria. *S. typhimurium* LT-2 and the LPS-deficient mutants derived from it were generously given to us by Hiroshi Nikaido of the University of California, Berkeley (16). The structures of the LPS of the parent and each mutant are given in Fig. 1. Bacteria were grown with aeration at 37°C to mid-log phase (absorbancy at 650 nm, 0.5; ca. 5×10^8 colony-forming units per ml) in Trypticase soy broth, harvested, and diluted as previously described (13). Bacteria were periodically checked with crystal violet for their degree of roughness (16).

Bactericidal assays. Unless noted otherwise, bacteria (2×10^3 to 4×10^3 colony-forming units per ml) were incubated for 1 h at 37°C with the appropriate granule fraction(s) in a total of 0.2 ml containing 0.5% tryptone plus 0.5% NaCl (pH 7) as described previously (13). Protein content of granule fractions was measured by the method of Lowry et al. (7), and appropriate amounts of extract were added to the bactericidal mixture to yield the concentrations indicated in the tables and figures. After incubation, 0.1 ml of the mixture was spread on Trypticase soy agar plates, incubated overnight at 37°C, and counted for colony-forming units.

Enzyme and protein quantitation. Lysozyme was measured by following the decrease in absorbancy of a suspension of *Micrococcus lysodeiktikus*, as described by Shugar (15), using human milk lysozyme purified in this lab by the method of Parry et al. (11) as a standard. Lactoferrin was measured by radial immunodiffusion by the method of Mancini et al. (8), using human milk lactoferrin purified in this lab by the method of Querinjean et al. (12) as a standard. Myeloperoxidase was measured by observing the reduction of *o*-dianisidine as described by Worthington (20), using human leukemic neutrophil myeloperoxi-

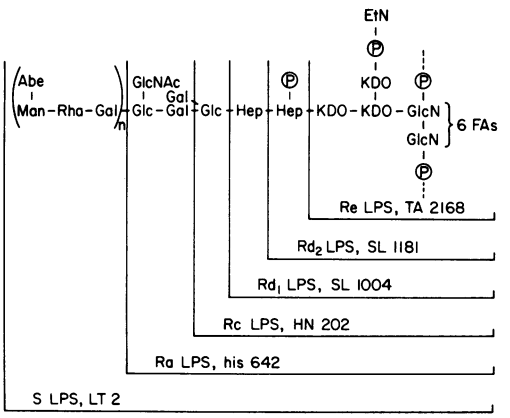

FIG. 1. *Chemical composition of the LPS of S. typhimurium LT-2 and its rough mutants. The type of rough mutant (Ra through Re) and the strain number are printed on the horizontal lines. Abe, Abequose; Man, D-mannose; Rha, L-rhamnose; Gal, D-galactose; GlcN, D-glucosamine; Hep, L-glycero-D-manno-heptose; KDO, 3-deoxy-D-mannooctulosonic acid; EtN, ethanolamine; P, phosphate; FA, fatty acids; and Ac, acetyl.*

dase purified in this lab by a modification of the method of Olsson et al. (10) as a standard. Protein was determined by the method of Lowry et al. (7), using crystalline egg while lysozyme as a standard.

Reagents. Ficoll, Triton X-100, *o*-dianisidine (free base), and egg white lysozyme (3× crystallized) were obtained from Sigma Chemical Co., St. Louis, Mo. Tryptone and lysozyme substrate (*M. lysodeikticus*) were obtained from Difco Laboratories, Detroit, Mich. Trypticase soy broth was purchased from BBL, Cockeysville, Md. Hypaque (Na, 50%) was obtained from Winthrop Laboratories. Human milk for the isolation of lactoferrin and lysozyme was generously supplied by the local La Leche League.

RESULTS

Composition of granule extracts. The presence of some characteristic proteins was measured in the phosphate buffered saline-dialyzed granule extracts (Table 1). The proteins measured were myeloperoxidase, found only in heavy and light azurophil granules (1, 5, 17, 19); lactoferrin, found only in specific granules (6); and lysozyme, found in all three granules (1, 5, 17, 19). There is no enzyme marker yet agreed upon for the tertiary granules which, in our sucrose density gradients, sediment just above, and are not well separated from, the specific granules. Using the above markers as indicators of granule purity, we showed that the specific granules were contaminated only about 5% by light azurophil granules, the light azurophil granules were contaminated about 18% by specific granules, and the heavy azurophil granules were not contaminated by specific granules. Since there are no markers to differentiate between light and heavy azurophil granules, it was not possible to determine their relative purity. Between 70 and 100% of each of the above proteins was extracted from whole granules by the acetate procedure used in this study, and it is therefore likely that the extracts used in this study were representative of the contents of whole neutrophil granules. Proteins in whole granules were measured in the presence of 0.05% Triton X-100, which yielded the highest values (data not shown).

Bactericidal activity of azurophil and specific granule extracts toward LPS mutants. The assay used in these studies differentiated between bactericidal and bacteriostatic activity. After 1 h of incubation, control bacteria under the assay conditions doubled their colony-forming units; therefore, if, after 1 h of incubation, experimental bacteria showed 50% of the colony-forming units of controls, then they had not grown, i.e., they were under bacteriostatic conditions. If after 1 h of incubation the percentage of colony-forming units was <50%, then it was considered bactericidal. To avoid confusion and misinterpretation of results, data are reported as "percent viable" bacteria after 1 h of incubation. Bacteria treated with extracts that were bacteriostatic at 1 h "grew out" after 2 h of incubation, i.e., overcame the bacteriostatic activity of the granule extracts.

Varying amounts of specific, light azurophil, heavy azurophil, or mixed granule extracts were incubated for 1 h at 37°C with *S. typhimurium* LT-2 and its LPS mutants (Fig. 2). The less carbohydrate that the bacteria possessed in their LPS, the more susceptible they were to the bactericidal and bacteriostatic activity of all extracts tested. Specific granules were bactericidal only for the Re mutant and only at concentrations ≥150 μg/ml. Extracts of heavy or light azurophil granules were similar to mixed granule extracts in their bactericidal and bacteriostatic activity. These granules were only bacteriostatic or slightly bactericidal for the smooth parent and the Ra mutant, but they were increasingly bactericidal for the Rc through Re mutants. There was a continuous increase in susceptibility to the bactericidal activity of the extracts as the mutants became rougher, with an apparent transition from a bacteriostatic to a bactericidal response as the *S. typhimurium* lost its core polysaccharides (from Ra to Rc or from Rc to Rd). The Ra mutant, containing no O antigen, was only slightly more susceptible than the S parent to the bacteriostatic action of all extracts tested.

Bactericidal activity of boiled granule extracts. Heating any of the granule extracts in a boiling-water bath for 10 min destroyed all of their bactericidal activity. The heated extracts remained bacteriostatic for the deep rough mutants (Rd_2 and Re; Fig. 3). Boiling for an additional 20 min did not destroy the residual bacteriostatic activity against the deep rough mutants.

Effect of pH on bactericidal activity. Bacteria were incubated for 1 h with different granule extracts in the Tryptone-NaCl incubation

TABLE 1. *Composition of phosphate buffered saline-dialyzed granule extracts*[a]

Granule extract	Protein (μg)	Myeloperoxidase (μg)	Lysozyme (μg)	Lactoferrin (μg)
Specific	8,350 (66)	87 (88)	1,400 (98)	1,450 (84)
Light azurophil	8,540 (88)	1,770 (99)	1,100 (100)	376 (100)
Heavy azurophil	15,525 (84)	1,210 (98)	1,260 (100)	0

[a] Numbers in parentheses refer to the percent of the protein that was extracted from whole granules. These data were obtained from 4.4×10^9 purified neutrophils.

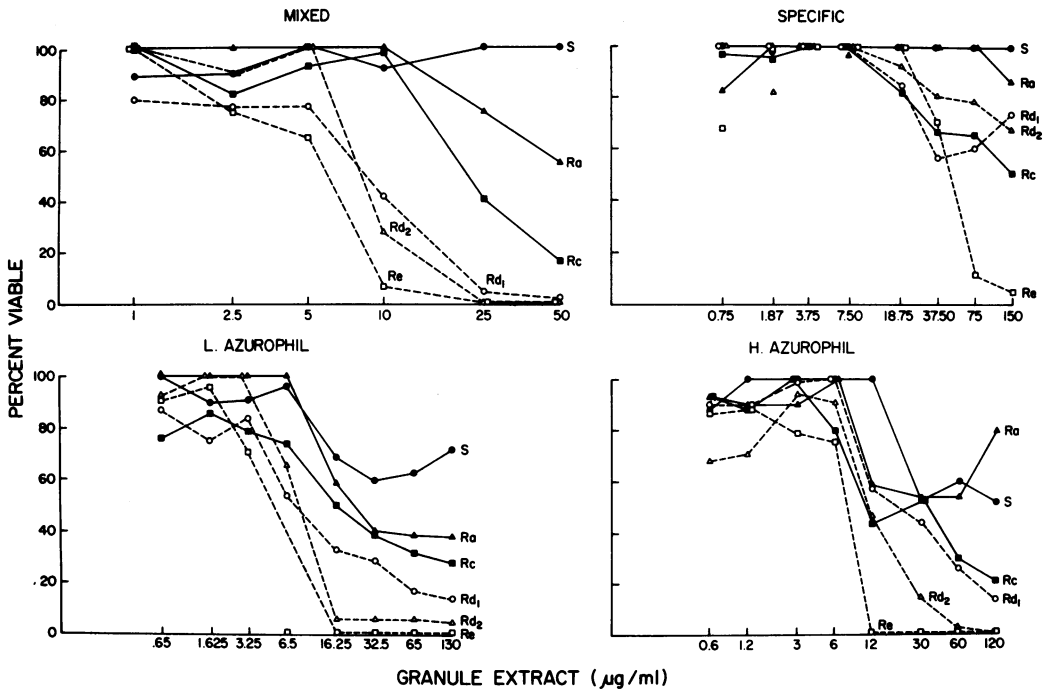

FIG. 2. *Effect of granule extracts on S. typhimurium LT-2 and its LPS mutants. Mixed extract was of a pellet (20,000 × g for 20 min) of a homogenized PMN supernatant (126 × g for 15 min). Specific, light (L) azurophil, and heavy (H) azurophil extracts were of pellets of appropriately diluted sucrose density gradient fractions (100,000 × g for 30 min). See legend to Fig. 1 for abbreviations and LPS structure.*

medium to which was added potassium phosphate buffer to 0.05 M at the appropriate pH. "Percent viable" bacteria was computed from controls performed at the respective pH's. Phosphate buffer neither inhibited nor stimulated growth at any pH tested. Mixed, heavy azurophil, and light azurophil granule extracts showed similar activities in that they killed the smooth *S. typhimurium* (S and Ra) best at pH 5, killed the rough *S. typhimurium* (Rd_2 and Re) best at pH 8, and killed the Rc and Rd_1 mutants to the same degree at pH 5 to 8 (Fig. 4). Specific granule extracts were bacteriostatic for Rd_2 mutants best at acid pH (5 to 6), whereas they killed the Re mutant only at pH 7 to 8.

Bactericidal activity at different incubation temperatures. Bacteria (S, Ra through Re) were killed best when incubated with extracts at 37°C. Little or no killing was observed at incubation temperatures of 2 to 3°C (ice), with intermediate bactericidal activity observed at 22°C (Fig. 5). The lack of bactericidal activity seen after incubation at 2 to 3°C was observed only if the bacteria were plated immediately after incubation at that temperature. If the bacteria were allowed to come to room temperature before plating, some killing was observed. With this information in mind, the following experiment was performed.

Bacteria (1×10^6 to 2×10^6 colony-forming units per ml) were incubated with mixed granule extract (70 μg/ml) on ice for 1 h, washed once in cold (2 to 3°C) phosphate-buffered saline, and suspended and diluted in warm (37°C) tryptone-NaCl medium without granule extract. The bacteria were then incubated at 37°C for additional time and plated. Bactericidal activity was observed after incubation in the absence of granule extract at 37°C following preincubation with granule extract at 2 to 3°C (Table 2). This activity was similar to the bactericidal activity observed when the bacteria were incubated at 37°C with mixed granule extract (see Fig. 2, 4, or 5 for comparison).

DISCUSSION

In this report, we presented data showing that azurophil granule extracts were much more bacteriostatic and bactericidal than were specific granule extracts. No significant differences were observed between the bactericidal activities of the two different azurophil granule populations. Mixtures containing both azurophil and specific granule extracts killed little or no better than

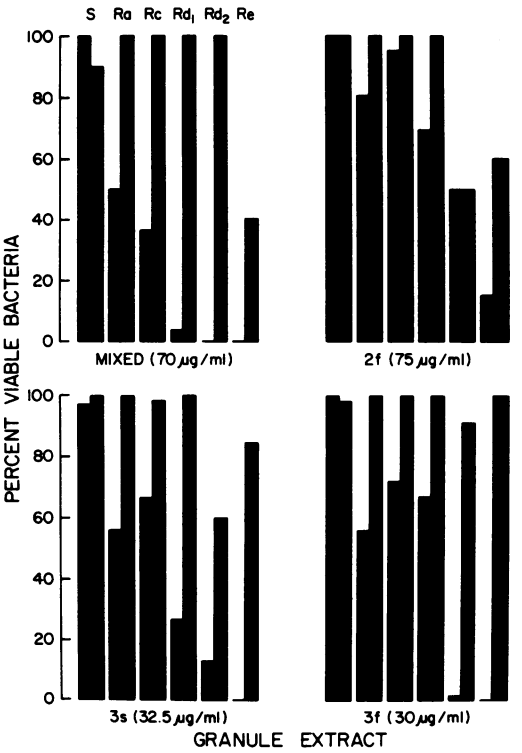

FIG. 3. *Effect of boiled granule extracts on S. typhimurium LT-2 and its LPS mutants. The left-hand bar of each pair is the control (not boiled) extract, and the right-hand bar is boiled extract (10 min). 2f, Specific granules; 3s, light azurophil granules; 3f, heavy azurophil granules. See legend to Fig. 1 for abbreviations and LPS structure.*

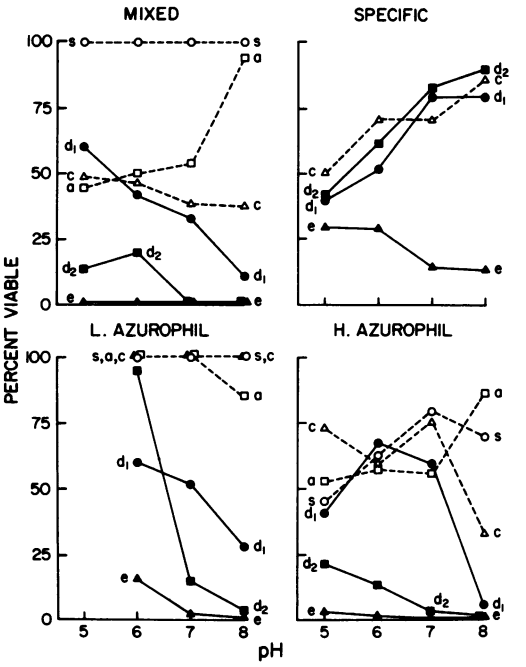

FIG. 4. *Effect of pH on the bactericidal and bacteriostatic activity of granule extracts on S. typhimurium LT-2 and its mutants. Mixed, 77.5 μg/ml; specific, 112.5 μg/ml; light azurophil, 97.5 μg/ml; heavy azurophil, 77.5 μg/ml. See legend to Fig. 1 for abbreviations and LPS structure.*

those containing azurophil granule extracts alone. Specific granule extracts were bacteriostatic, not bactericidal, for all bacteria except the deep rough (Re) mutant. These results indicate the minor role that specific granules play in PMN bactericidal activity, and they have been supported in this laboratory by in vitro phagocytosis experiments. Human PMN depleted of specific granules by pretreatment with phorbol myristate acetate showed no bactericidal defect when challenged with low numbers of *Escherichia coli* (less than 10 bacteria per PMN). PMN did show decreased bactericidal activity when challenged with larger numbers of *E. coli* (P. Wang-Iverson et al., submitted for publication).

The bactericidal assay used in this report has been defined in more detail in an earlier publication (13). Briefly, bacteria grown with aeration to early- or mid-log phase were used because they are more sensitive to the bactericidal activity of granule extracts than are bacteria grown under static conditions to stationary phase. Bacteria were incubated with granule extracts at 37°C for 1 h in a medium containing 0.5% NaCl and 0.5% tryptone (pH 7). These conditions were chosen because bactericidal activity was greater at 37°C than at lower temperatures, bactericidal activity was usually complete within 1 h at 37°C, and bacteria were killed better when incubated in growth conditions (e.g., tryptone) than when incubated in nongrowth conditions (e.g., phosphate-buffered saline). None of the bacteria clumped during incubation with or without granule extract, as determined by light microscopic examination of Gram-stained samples.

Rough *S. typhimurium* was killed better than smooth *S. typhimurium* when incubated with any of the extracts tested. As the bacteria lost their LPS carbohydrate, they became gradually more sensitive to the bactericidal activity of the extracts. No point was evident where there was a quantum jump in sensitivity to the bactericidal activity of mixed granule extracts. However, S (smooth) and Ra bacteria seemed to be only growth inhibited by even high concentrations of granule extracts, whereas Rc through Re bacteria were killed by the same extracts at lower concentrations. That is, a transition of bacteriostatic to bactericidal response was observed

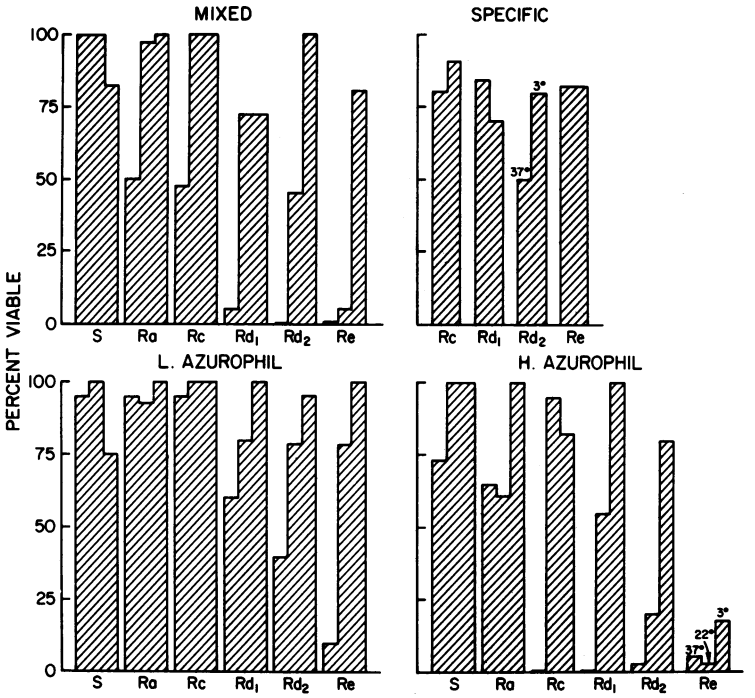

FIG. 5. *Effect of temperature on the bacteriostatic and bactericidal activity of granule extracts on S. typhimurium LT-2 and its LPS mutants. Mixed, 75.0 μg/ml; specific, 100.0 μg/ml; light azurophil, 81.5 μg/ml; heavy azurophil, 120.0 μg/ml. See legend to Fig. 1 for abbreviations and LPS structure.*

TABLE 2. *Response of S. typhimurium exposed to mixed granule extract*[a]

S. typhimurium LPS type	Viable bacteria (%) at 37°C after:	
	10 min	60 min
S	97	87
Ra	92	85
Rc	38	35
Rd_1	42	31
Rd_2	10	7
Re	27	2

[a] Bacteria (1×10^6 to 2×10^6) were exposed to 70 μg of mixed granule extract per ml at 3°C, then incubated at 37°C with no extract.

with loss of core polysaccharides. Using guinea pig neutrophil mixed granule extracts, Friedberg and Shilo observed a sharp increase in killing when *S. typhimurium* lost any part of its core polysaccharide, with no increase in sensitivity upon loss of O antigen (3, 4). Tagesson and Stendahl obtained similar results using rabbit granule extracts with *S. typhimurium* (18). We observed, at each step, increases in sensitivity, in loss of O antigen, loss of core polysaccharide, and loss of heptoses. The differences observed between the bactericidal activity of animal neutrophil granule extracts and human neutrophil granule extracts can be explained in several ways. Our incubations were performed in a growth medium (tryptone), whereas the work of others was done in a nongrowth medium (phosphate or citrate buffer). In our study, mid-log phase bacteria were used, whereas the bacteria used by others were late-log or resting. Of primary importance is that human neutrophil granules contain both quantitatively and qualitatively different bactericidal and degradative proteins than do animal neutrophil granules (2).

Odeberg and Olsson showed that azurophil granules contain cationic proteins (possessing neutral protease activity) that are bactericidal due to their cationic, as opposed to their enzymatic, properties; i.e., boiled cationic proteins were as bactericidal as nonboiled (9). In the present study, boiled azurophil granule extracts retained only their bacteriostatic activity for the deep rough (Rd and Re) mutants, retaining no bactericidal activity. This suggests that the majority of killing observed with azurophil granule extracts might not be due to the cationic nature of proteins found in the extract but to some heat-labile enzymatic activity.

In an attempt to investigate the mechanisms of killing by the granule extracts for smooth or rough bacteria, the bactericidal assays were done

at varying pH values. We showed previously that mixed granule extracts killed smooth (S) bacteria best at pH 5 and rough (Re) bacteria best at pH 7 to 8 (13). In this report we expand this to show that intermediate rough bacteria (Rc) are killed to the same degree at pH 5 through 8 by azurophil or mixed granule extracts. There thus appears to be a gradual transition. The bacteriostatic pH optimum of specific granules for rough bacteria (Rd and Re) followed a similar pattern. The idea that rough bacteria are more sensitive to granule extract simply because they have lost some of their protective coat might thus be too simplistic. There appears to be a gradual change in the enzyme system(s) involved in the bactericidal event(s) as the bacteria lose LPS and thus change their surface components and surface character.

Further indications as to what is occurring in the microenvironment of the interior of the phagolysosome are indicated by the temperature and "sticking" experiments. Bacteria that were exposed to granule extracts at 2°C and then immediately plated were neither killed nor growth inhibited, indicating that no permanent damage was inflicted upon the bacteria under these conditions. The results also suggest that Trypticase soy media inhibited the action of bactericidal proteins that stuck to the bacteria. If this were not the case, killing should have occurred, as indicated in the following experiment. When bacteria were treated with mixed granule extract at 2°C, then washed free of extract and further incubated at 37°C, killing was observed. These results suggest a number of things, the most obvious of which is that bactericidal proteins stuck to the bacteria strongly enough so that they were not washed off during normal Vortex mixing and centrifugation. They also support the evidence that killing does not occur at 2°C. Whether this is due to a decrease in bacterial metabolism, bactericidal protein activity, or both, is being investigated.

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