INFECTION AND IMMUNITY, Aug. 1979, p. 597-602
0019-9567/79/08-0597/06$02.00/0

Vol. 25, No. 2

Bactericidal Activity of Fractionated Granule Contents from Human Polymorphonuclear Leukocytes: Antagonism of Granule Cationic Proteins by Lipopolysaccharide

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Received for publication 21 May 1979

Granule extracts from human polymorphonuclear leukocytes (PMN) were prepared with 0.2 M (pH 4.0) acetate. A fraction (valley AB) with distinctive bactericidal activity against cell wall mutants of *Salmonella typhimurium* LT-2 was obtained after fractionation of the granule extracts by Sephadex G-100 column chromatography. The smooth parent LT-2 strain was less sensitive to the bactericidal action. Susceptibility of the rough mutants to bactericidal action increased as sugar residues decreased in the lipopolysaccharide (LPS) (Re > Rd_2 > Rd_1 > Rc > Ra). Cationic protein(s) responsible for bactericidal activity could be selectively removed from the fraction by absorption with whole LT-2 cells or purified LPS. Loss of cationic protein species was confirmed by cationic polyacrylamide gel electrophoresis. Purified LPS from LT-2 or the deep rough mutant TA2168 inhibited the antimicrobial activity of the killing fraction in in vitro assays. A minor protein species (vAB_1) from the valley AB fraction had an apparent molecular weight of 36,000 to 37,000 and represented a major bactericidal activity of the fraction. Small amounts of the isolated vAB_1 protein were bactericidal for the smooth parent LT-2 strain.

A variety of antimicrobial substances and hydrolytic enzymes are associated with the cytoplasmic granules of polymorphonuclear leukocytes (PMN) (14, 16). The bactericidal mechanisms associated with these granule components have been explained on the basis of two classes of mechanisms, those dependent on H_2O_2 and other energy states of oxygen and those independent of them (15). These cytoplasmic granules can be obtained in purified form, and the contents can be extracted with dilute acid (7). In a previous report we described the separation of PMN granule extracts by Sephadex G-100 column chromatography that yielded fractions possessing bactericidal activity against a series of progressively rough lipopolysaccharide (LPS) mutants of *Salmonella typhimurium* LT-2 (7). A single component of this valley AB fraction, representing a major bactericidal activity, subsequently has been identified. The identification of this protein (vAB_1), as well as factors influencing its interaction with *S. typhimurium* LT-2 and a series of progressively rough LPS mutants derived from LT-2, is the subject of this report.

MATERIALS AND METHODS

Purification of PMN. Human neutrophils were obtained in large quantity by leukapheresis 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 (9, 12).

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 Potter Elvehjem glass 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 863 mg of granule protein was chromatographed by previously published procedures (8). Fractions were pooled, concentrated by ultrafiltration (Amicon UM-05 filter) to 4 to 6 mg/ml, and dialyzed against phosphate-buffered saline (PBS) (pH 7.0) before use in bactericidal assays.

Bacteria. *S. typhimurium* LT-2 and the LPS-deficient mutants *his*-642 (Ra), HN202 (Rc), SL1004 (Rd_1), SL1181 (Rd_2), and TA2168 (Re) derived from it

have been described previously (9, 13).

Bactericidal assays. Bactericidal assays were done by the method of Rest et al. (9, 10) in a total of 0.2 ml of tryptone-NaCl (pH 7.0) in the wells of a small plastic Linbro tray (Flow Laboratories). Assay mixtures contained 2×10^3 to 4×10^3 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 (BBL Microbiology Systems) soy agar plates and colony-forming units were counted at 18 to 24 h. Results are expressed as the 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.

PAGE. Electrophoresis in 10% polyacrylamide gels (PAGE) was performed for cationic enzyme samples by the method described by Gabriel (3). Samples containing 160 μg of protein were dialyzed against β-alanine (3.12 g/liter)-acetic acid (0.8 ml/liter) running buffer (pH 4.5) and were loaded with 6% final concentration sucrose to the top of the gels (4 by 50 mm). Samples were allowed to enter the stacking gel at 1.5 mA/gel for 15 min. Electrophoresis was then conducted at 3.0 mA/gel for approximately 2 h or until the tracking dye (methyl green) reached the end of the gel. Proteins in gels not used for bactericidal assays were visualized with Coomassie brilliant blue G250 by the method of Diezel et al. (2). Gels were scanned with a Gilford model 2000 recording spectrophotometer equipped with a 2520 gel scanner. Gel scan rate was 2.0 cm/min with chart paper travel set at 5.08 cm/min. Absorbance at 595 nm was recorded with full-scale deflection set at 1.0. Gels used for bactericidal assays were sliced into 2-mm fractions with a Bio-Rad model 190 gel slicer. Protein in individual slices was eluted with 100 μl of sterile PBS, and each sample was dialyzed extensively in a multicavity microdialyzer (Preiser Scientific) to remove any residual β-alanine. Protein recovered from the gel slices was estimated by the fluorometric method described by Bohlen et al. (1) using chicken egg white lysozyme as standard and concentrated by ultrafiltration (Amicon UM-05 filter) when necessary for further electrophoresis analysis.

Reductive sodium dodecyl sulfate (SDS)-PAGE was performed on protein recovered from cationic PAGE gels according to the method of Weber and Osborne (17). Approximately 20 μg of protein was reduced with β-mercaptoethanol in the presence of SDS before electrophoresis in 0.1% SDS-5% polyacrylamide gels at 8 mA/gel. For molecular weight determination, RNase, chymotrypsinogen, and ovalbumin (Pharmacia) were run, and their mobilities were plotted as a function of log molecular weight.

Purification of LPS. Bacterial LPS was extracted from *S. typhimurium* LT-2 by the phenol-water method of Westphal and Jann (18). The type Re LPS from *S. typhimurium* TA2168 was extracted by the phenol-chloroform-petroleum ether procedure of Galanos et al. (4). LPS was suspended in PBS and sterilized by filtration (0.45 μm) before addition to bacterial assays. LPS was quantified by estimation of 2-keto-3-deoxyoctonate by a method described previously (5).

Adsorption of valley AB protein to whole cells. *S. typhimurium* LT-2 was grown overnight in 100 ml of Trypticase soy broth with shaking at 37°C. A fresh culture (100 ml) was started the next day with 1.0 ml of the overnight culture. Cells were incubated at 37°C with shaking for 3 h (0.6 at an optical density at 650 nm [OD_{650}]). A 60-ml portion of the culture was collected by centrifugation at 8,000 rpm for 10 min at 4°C (Sorvall SS-34), washed once with sterile PBS (pH 7.0), and resuspended in PBS to 1.0 OD_{650}. A 500-μg portion of valley AB protein was added to 5 ml of cells and incubated for 1 h at 22°C. After incubation, cells were removed by centrifugation at 8,000 rpm for 10 min at 4°C and washed once with PBS. The supernatants and wash fluids were pooled and passed through a 0.45-μm membrane filter (Millipore Corp.) to remove any remaining cells. Supernatant fluids were concentrated by ultrafiltration (Amicon UM-05 filter) to 4 to 6 mg/ml and used directly in bactericidal assays or dialyzed against cationic PAGE running buffer for electrophoretic analysis.

Protein. Protein was measured by the method of Lowry et al. (6) with chicken egg white lysozyme as standard.

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

RESULTS

PAGE of Sephadex G-100 fractions. Human PMN granule extracts separated by Sephadex G-100 column chromatography (Fig. 1) resolve into four protein peaks (A, B, C, D) which flank three valley regions of low protein concentration (AB, BC, CD). Figure 2 shows cationic PAGE patterns rendered by proteins from pooled fractions from these peaks and valleys. The valley AB region contains a number of different protein species and has potent bactericidal activity against a series of mutants of *S. typhimurium* LT-2 that carry progressively shorter polysaccharide chains in their LPS. This

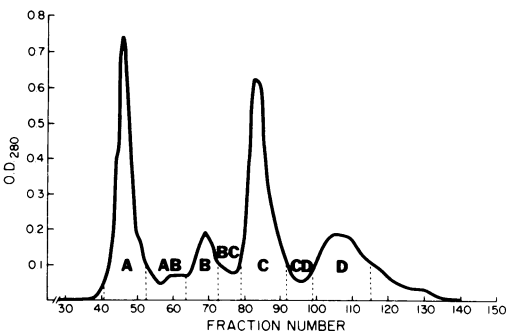


FIG. 1. *Elution profile obtained for Sephadex G-100 column chromatographic separation of human PMN granule protein. Elution from the column (5 by 90 cm) was with 0.2 M sodium acetate buffer (pH 4.0). Fraction volumes were 16.0 ml. Fractions 1 to 38 represent the void volume. All operations were conducted at 4°C.*

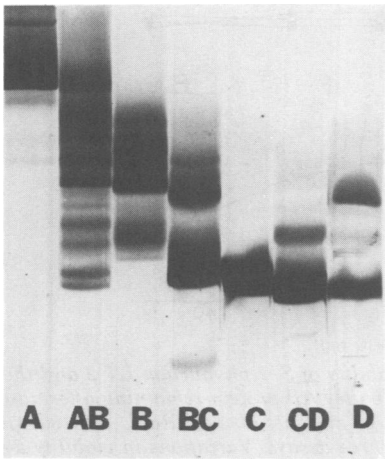


FIG. 2. *Cationic PAGE of 160 μg each of peak A, valley AB, peak B, valley BC, peak C, valley CD, and peak D from the Sephadex G-100 column chromatographic separation of human PMN granule extract.*

activity is not duplicated by mixtures of peaks A and B (7).

Binding of valley AB protein to *S. typhimurium* LT-2 cells. Whole cells of *S. typhimurium* LT-2 were incubated with valley AB protein for 1 h. When the cells were removed by centrifugation, followed by filtration, the resultant supernatant fluid showed a loss of proteins from the valley AB fraction. Figure 3 shows the cationic PAGE profile of proteins found in the valley AB fraction before (a) and after (b) absorption by LT-2 cells. The valley AB protein remained bound to the LT-2 cells during washing with PBS, suggesting a strong attachment to the outer membrane of LT-2. Similar experiments with the remaining Sephadex G-100 fractions indicated that only valley AB protein demonstrated this binding to LT-2 cells. The valley AB protein remaining in the supernatant fluid was examined for bactericidal activity. Figure 4 compares the effect of increasing concentrations of valley AB protein on LT-2 and its LPS mutants before (A) and after (B) absorption by whole LT-2 cells. The rough mutants with types R_a, Rd_1, and Re LPS were susceptible to valley AB protein at concentrations as low as 2.0 μg per assay volume (A). When concentrations were increased, the LT-2 parent strain also showed susceptibility to the bactericidal activity of the valley AB fraction. However, the valley AB proteins remaining in the supernatant after absorption to whole LT-2 cells did not demonstrate potent bactericidal activity (B). LT-2, as well as *his*-642 and SL1004, was not sensitive to the remaining protein. Slight activity was demonstrated against the deep rough TA2168 strain with type Re LPS.

Effect of bacterial LPS on valley AB protein. When purified LPS from *S. typhimurium* LT-2 (200 μg) was mixed with valley AB protein (200 μg) and subjected to cationic PAGE, the results were identical to those seen in Fig. 3. Therefore, to examine the effect of LPS on the binding of valley AB protein and the possible role of LPS in protecting *S. typhimurium* from the bactericidal action of the valley AB fraction, purified LPS from LT-2 was added to in vitro bactericidal assays containing 10 μg of valley AB protein (Fig. 5). Substantial increases in rough mutant cell viability were observed with increasing concentrations of LT-2 LPS. The amount of LT-2 LPS required to fully protect the rough mutant cells from the bactericidal activity of the valley AB fraction appeared to be related to the degree of roughness of the mutant. As little as 0.4 μg of LT-2 LPS protected *his*-642 against 10 μg of valley AB protein. However, as much as 1 to 2 μg was required to fully protect the SL1004 and TA2168 strains. Figure 5 also shows that LPS isolated from the TA2168 mutant could protect the mutant series from the bactericidal effects of valley AB protein. However, a 50-fold-greater concentration of this deep rough LPS was necessary to achieve the levels of viability observed when LT-2 LPS was used.

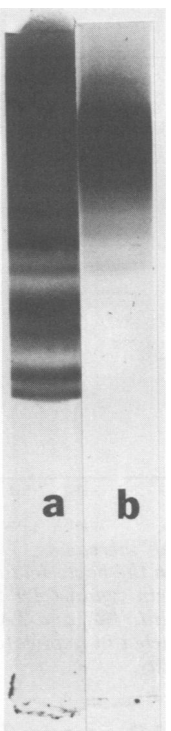


FIG. 3. *Cationic PAGE of 200 μg of valley AB protein before (a) and after (b) adsorption to whole cells of S. typhimurium LT-2.*

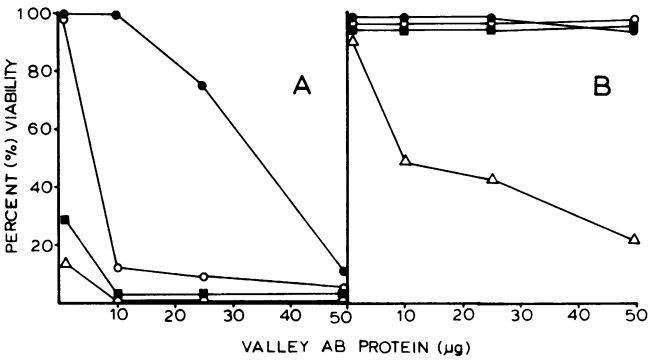
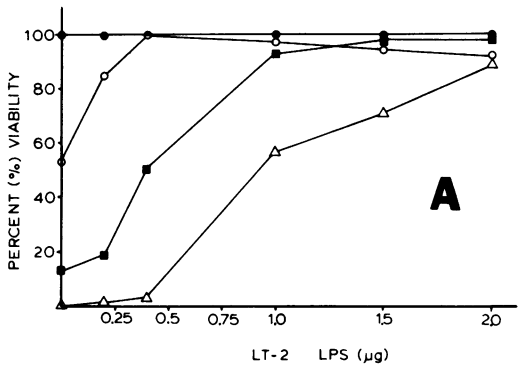


FIG. 4. *(A) Effect of increasing valley AB protein on the viability of S. typhimurium LT-2 and the rough mutants his 642, SL1004, and TA2168. (B) Effect of increasing valley AB protein remaining after absorption by whole LT-2 cells on the smooth parent LT-2 (●) and the rough mutants his 642 (Ra, ○), SL1004 (Rd_1, ■), and TA2168 (Re, △). Results shown are average values from three assays. Variations in viability were less than 5%.*



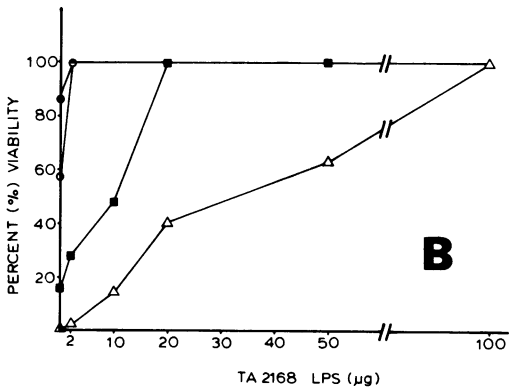


FIG. 5. *Effect of increasing LT-2 LPS (A) or TA2168 LPS (B) on the bactericidal activity of 10 μg of valley AB protein against LT-2 (S) (●), his-642 (Ra) (○), SL1004 (Rd_1) (■), and TA2168 (Re) (△). All assays were conducted in triplicate. Variability was less than 5% viability.*

Isolation of vAB_1 protein. The protein complement from the valley AB fraction was resolved by cationic PAGE. The gels were cut into 2.0-mm slices and the protein was allowed to elute from the gel slices overnight. The eluted proteins were not corrected for protein concentration and were tested directly for bactericidal activity against the smooth parent LT-2 and the LPS rough mutant series. The individual eluted proteins showed increasing activity against the mutant series as the degree of roughness increased. A major bactericidal activity centered around the area of the gel that contained a distinct protein species (slice 15) that was also active against the smooth LT-2 parent (Fig. 6). This protein represented only 3.0% of the total protein of the valley AB fraction yet showed reproducible bactericidal activity against the smooth parent at low protein concentrations. The protein of gel slice 15 was termed the vAB_1 protein.

Molecular weight determination of vAB_1. The protein demonstrating major bactericidal activity (slice 15) was eluted from the cationic PAGE gels and examined by SDS-PAGE. The relative mobility of vAB_1 on electrophoresis was compared with those of protein standards, and relative mobilities were plotted as a function of log molecular weight (Fig. 7). The vAB_1 protein had an apparent molecular weight of 36,000 to 37,000 and appeared as a homogeneous protein band in SDS-PAGE and cationic PAGE.

DISCUSSION

The valley AB fraction of acetate-extracted granule contents from human PMN possesses a potent bactericidal activity against outer-membrane LPS mutants of *S. typhimurium* LT-2. Of the bacteria tested, those demonstrating greater degrees of roughness were most sensitive to the action of valley AB protein (7). The parent and mutant strains possess different substrates on

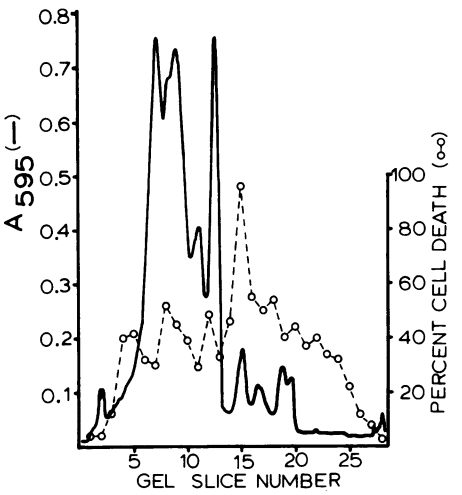


FIG. 6. *Localization of bactericidal activity of valley AB proteins resolved by cationic PAGE against S. typhimurium LT-2. Results shown indicate the average of three assays. Variation in bactericidal activity was less than 5% viability.*

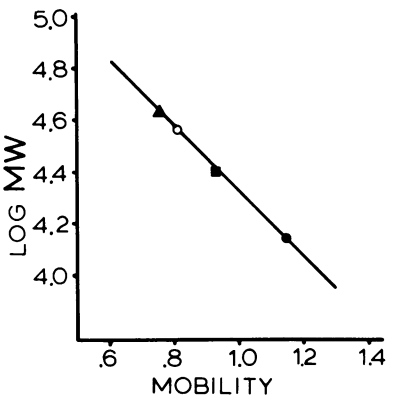


FIG. 7. *Molecular weight (MW) determination of vAB$_1$ protein* (○) *by SDS-PAGE. Molecular weight standards include ovalbumin* (▲), *chymotrypsin* (■), *and RNase* (●).

their surfaces and as such can be used as probes for detecting and defining different enzyme systems involved in the bactericidal process.

Because the smooth parent LT-2 was less sensitive to the bactericidal action of valley AB fraction, we examined the role of complete outer-membrane LPS in the binding of PMN cationic protein. We found that whole LT-2 cells could quantitatively remove certain proteins from the valley AB fraction. That these valley AB proteins were bound directly to outer-membrane LPS was further substantiated by the observations that purified LPS derived from LT-2 could also quantitatively remove protein species from the cationic PAGE gel profiles. Valley AB protein remaining after absorption by LT-2 cells or purified LPS demonstrated minimal bactericidal activity. The observed sensitivity of the deep rough TA2168 LPS mutant to unabsorbed valley AB protein could have been due to residual vAB$_1$ protein remaining in the fraction. Alternatively, the sensitivity may have been due to other potentially bactericidal protein species in the valley AB fraction that could only be recognized when the potent vAB$_1$ protein was removed.

Addition of purified LPS derived from LT-2 or TA2168 to bactericidal assays appeared to inhibit the bactericidal activity of valley AB protein. The amount of inhibition was dependent on the concentration of LPS added to assay mixtures and also on the quality of LPS added. The complete LT-2 LPS molecule was much more efficient in protecting cells from the bactericidal action of valley AB protein than was the TA2168 LPS. These data indicated that the amount and type of LPS present on the surface of the bacteria played an important role in resistance to bactericidal PMN granule proteins.

We utilized the differential sensitivity of rough mutants of *S. typhimurium* LT-2 to identify a specific bactericidal protein (vAB$_1$) responsible for a major bactericidal activity in the valley AB fraction. The vAB$_1$ protein comprised only 3% of the valley AB protein. This potent microbicidal protein had an apparent molecular weight of 36,000 to 37,000 and was bactericidal for the smooth parent LT-2 in concentrations as low as 4.5 μg of protein per ml.

The vAB$_1$ protein described represents a potent bactericidal constituent of the granules of human PMN. The microbicidal activity of vAB$_1$ in vivo, where concentrations in phagolysosomes are probably large compared with those in in vitro assay systems, where achievable concentrations are small, may moreover be augmented by other systems such as glycosidases (11) that render the bacterial surface more susceptible to its action. Further investigations are in progress to define the mechanism of bactericidal action of vAB$_1$ protein for *S. typhimurium* and other gram-negative bacteria.

ACKNOWLEDGMENTS

The excellent technical assistance of L. E. Martin and M. H. Cooney and expert clerical assistance of J. Goldman and M. K. Gatewood are gratefully acknowledged. We thank R. Twarog for use of the Gilford model 2000 recording spectrophotometer.

This work was supported by Public Health Service grants AI 13464 and AI 02430 from the National Institutes of Health and by Department of Energy grant 3628-9.

LITERATURE CITED

1. **Bohlen, P., S. Stein, W. Doirman, and S. Udenfriend.**

1973. Fluorometric assay of proteins in the nanogram range. Arch. Biochem. Biophys. **155**:213–220.

2. **Diezel, W., G. Kopperschlager, and E. Hofman.** 1972. An improved procedure for protein staining in polyacrylamide gels with a new type of Coomassie brilliant blue. Anal. Biochem. **48**:617–620.
3. **Gabriel, O.** 1971. Analytical disc gel electrophoresis. Methods Enzymol. **22**:565–578.
4. **Galanos, C., O. Luderitz, and O. Westphal.** 1969. A new method for the extraction of R. lipopolysaccharides. Eur. J. Biochem. **9**:245–249.
5. **Keleti, G., and W. H. Lederer.** 1974. Micro-methods for the biological sciences, p. 74–75. Van Nostrand Reinhold Company, New York.
6. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
7. **Modrzakowski, M. C., M. H. Cooney, L. E. Martin, and J. K. Spitznagel.** 1979. Bactericidal activity of fractionated granule contents from human polymorphonuclear leukocytes. Infect. Immun. **23**:587–591.
8. **Pryzwansky, K. B., L. E. Martin, and J. K. Spitznagel.** 1978. Immunocytochemical localization of myeloperoxidase, lactoferrin, lysozyme and neutral proteases in human monocytes and neutrophilic granulocytes. RES J. Reticuloendothel. Soc. **24**:295–310.
9. **Rest, R. F., M. H. Cooney, and J. K. Spitznagel.** 1977. Susceptibility of lipopolysaccharide mutants to the bactericidal action of human neutrophil lysosomal fractions. Infect. Immun. **16**:145–151.
10. **Rest, R. F., M. H. Cooney, and J. K. Spitznagel.** 1978. Bactericidal activity of specific and azurophil granules from human neutrophils: studies with outer-membrane mutants of *Salmonella typhimurium* LT-2. Infect. Immun. **19**:131–137.
11. **Rest, R. F., M. H. Cooney, and J. K. Spitznagel.** 1978. Subcellular distribution of glycosidases in human polymorphonuclear leukocytes. Biochem. J. **174**:53–59.
12. **Rest, R. F., and J. K. Spitznagel.** 1977. Subcellular distribution of superoxide dismutases in human neutrophils. Influence of myeloperoxidase on the measurement of superoxide dismutase activity. Biochem. J. **166**: 145–153.
13. **Smit, J., Y. Kamio, and H. Nikaido.** 1975. Outer membrane of *Salmonella typhimurium*: chemical analysis and freeze-fracture studies with lipopolysaccharide mutants. J. Bacteriol. **124**:942–958.
14. **Spitznagel, J. K.** 1975. Advances in the study of cytoplasmic granules of human neutrophilic polymorphonuclear leukocytes, p. 77–85. *In* J. A. Bellanti and D. H. Dayton (ed.), The phagocytic cell in host resistance. Raven Press, New York.
15. **Spitznagel, J. K.** 1975. Mechanisms of killing by polymorphonuclear leukocytes, p. 209–214. *In* D. Schlessinger (ed.), Microbiology—1975. American Society for Microbiology, Washington, D.C.
16. **Spitznagel, J. K.** 1977. Bactericidal mechanisms of the granulocyte, p. 103–131. *In* T. J. Greenwalt and G. A. Jamieson (ed.), The granulocyte: function and clinical utilization. Alan R. Liss, Inc., New York.
17. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**: 4406–4412.
18. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure, p. 83–91. *In* R. L. Whistler, J. N. Bemiller, and M. L. Wolfram (ed.), Methods in carbohydrate chemistry V. Academic Press, Inc., New York.