Purification and Characterization of a Potent Bactericidal and Membrane Active Protein from the Granules of Human Polymorphonuclear Leukocytes

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A potently bactericidal cationic protein that increases the envelope permeability of susceptible Gram-negative bacteria has been isolated from human leukocytes and purified to near homogeneity. The active fraction was extracted with 0.2 M sodium acetate (pH 4.0) from the granules of polymorphonuclear leukocytes obtained from normal individuals or from patients with chronic myelocytic leukemia. Mononuclear cells contained less than 30% of the activity extracted from polymorphonuclear cells. Successive chromatography of acid extracts on Sephadex G-75 and Bio-Rex 70 purified 40-fold a protein fraction that appeared to be at least 95 to 98% pure as judged by rechromatography on Sephadex G-100, isoelectric focusing, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This protein has an isoelectric point at pH 9.8 and an apparent molecular weight of 58,000 to 60,000, estimated by gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The purified protein exhibits no protease, lysozyme, myeloperoxidase, or phospholipase A activity and may, therefore, be a noncatalytic bactericidal protein. Killing by the purified protein of several strains of Escherichia coli and Salmonella typhimurium occurs with only slight inhibition of bacterial protein synthesis, but is accompanied by an almost immediate increase in microbial envelope permeability (1, 3). The results suggest that at neutral pH, it is the principal entry of actinomycin D can be measured by the inhibitory effect of the antibiotic on protein (or RNA) synthesis. Both bactericidal and permeability increasing activities are maximal at pH 7.0, inhibited by Mg²⁺, Ca³⁺, or heparin, and inactivated by heating at 80°C for 10 min.

"Rough" strains of E. coli and S. typhimurium are most sensitive to the biological effects (0.5 to 2.0 μg of protein (10 to 40 ng) produce >90% bactericidal and permeability effects on 10⁷ bacteria/ml); "smooth" strains are less sensitive (2 to 20 μg), and several species of Gram-positive bacteria as well as two strains of the fungus Candida are unaffected by 20 to 100 μg of protein.

This bactericidal protein is at least 20 to 50 times more potent towards susceptible bacteria than any other fraction derived from leukocyte granule extracts, including the previously described antimicrobial cationic proteins of human polymorphonuclear leukocytes. However, the bactericidal potency and specificity as well as the molecular properties of the bactericidal and permeability-increasing protein recently isolated from rabbit polymorphonuclear leukocytes and of the human leukocyte protein described herein, are remarkably similar.

We have recently reported the purification of a previously unidentified cationic protein fraction from rabbit polymorphonuclear leukocytes that is potently bactericidal towards Escherichia coli and several other Gram-negative bacteria (1, 2). Extensive and irreversible loss of the bacteria's ability to multiply is initiated by this fraction within 5 min of inoculation with neither demonstrable degradation of bacterial macromolecular constituents nor inhibition of their biosynthesis. Killing is accompanied, however, by an almost immediate, discrete increase in microbial envelope permeability (1, 3). The potency of the purified fraction towards susceptible microorganisms (10 nM produces more than 90% killing of 10⁷ bacteria/ml) suggests that it may be an important component of the antibacterial arsenal of polymorphonuclear leukocytes.

In the present study we describe the purification of a cationic protein from human polymorphonuclear leukocytes that possesses very similar bactericidal and permeability-increasing activities. The potent antimicrobial activity of this protein appears to be specific for Gram-negative bacteria and the results suggest that, at neutral pH, it is the principal antibacterial agent present in crude acid extracts of human polymorphonuclear leukocyte granules towards this group of microorganisms.

EXPERIMENTAL PROCEDURES

Materials

Sephadex G-75 (superfine), Sephadex G-100 (medium), and blue dextran 2000 were purchased from Pharmacia Fine Chemicals, AB.

1 Unpublished observation.
Bio-Rex 70 (200 to 400 mesh) was obtained from Bio-Rad Laboratories. Ampholine mixtures with pH range of 7 to 9 and 9 to 11 (Ampholine 1809-136 and 1809-146) were obtained from LKB-Produkt AB. Acrylamide and N,N'-methylenebisacrylamide were obtained from Eastman Kodak Co. and sodium dodecyl sulfate (sodium lauryl sulfate) from Pierce Chemical Company. Sucrose (ultrapure, density 1.620 g/ml) was obtained fromามPARA Chemicals and Isopaque from Winthrop Laboratories. Bacto-peptone (Sigma Co.). Dextran (clinical grade, average molecular weight 73,200) was from Sigma. Ficoll 400 was obtained from Pharmacia (Ampholine 1809-136 and 1809-146) were obtained from LKB-Products. Sucrose (ultrapure, density 1.620 g/ml) was obtained from International Chemical and Nuclear Corporation.

Leukocytes

Leukocytes were obtained from heparin-treated (143 U.S. units/10 ml) peripheral blood collected by venapuncture after informed consent from healthy donors and patients with chronic myelocytic leukemia. Populations of polymorphonuclear leukocytes were obtained in two ways. 1) Polymorphonuclear leukocytes were prepared by the differential centrifugation procedure, followed by centrifugation in an Isopaque-Ficoll gradient according to Boyum (4). The leukocyte-rich plasma from leukemic blood was first diluted with Krebs-Ringer buffer to a concentration of 10,000 to 20,000 cells/μl before layering on the Isopaque-Ficoll mixture. Such polymorphonuclear leukocytes prepared in this way contained about 10% mononuclear cells as judged by differential cell count of Wright-stained smears. Mononuclear cell fractions from the Isopaque-Ficoll gradient were contaminated with about 10% polymorphonuclear leukocytes. All cell fractions were washed twice in Krebs-Ringer phosphate before use. 2) To procure sufficient numbers of polymorphonuclear leukocytes for purification on a larger scale the Isopaque-Ficoll gradient is not suitable. For this reason the leukocyte-rich plasma obtained by leukopheresis of 400 ml of venous blood of a patient with chronic myelocytic leukemia (5) was sedimented directly at 1000 g for 30 min. 

Preparation of Granule-rich Fraction

To obtain granules the leukocytes were resuspended in 0.34 M sucrose (2 x 10^8 cells/ml) and homogenized at 0° in a Potter-Elvehjem glass homogenizer equipped with a motor-driven Teflon pestle. A granule-rich fraction was obtained by differential centrifugation of the homogenate as previously described (6).

Acid Extraction of Bactericidal and Permeability-increasing Activities from Leukocyte Homogenates or Granule-rich Fractions

Three acid-extraction procedures that have been used by others to extract leukocyte activities (1, 7, 6) were compared.

Sulfuric Acid Extraction of Whole Cells—Polymorphonuclear leukocytes or mononuclear cells, sedimented by centrifugation and resuspended in distilled water (3 x 10^8 cells/ml), were homogenized vigorously and extracted with 0.16 M H2SO4 for 30 min at 0° with periodic shaking. Neutralization of the extract by prolonged dialysis and removal of dense precipitates that form during dialysis by centrifugation at 23,000 g for 20 min were carried out as previously described (1) except that dialysis was against 2 mM Tris/HCl, pH 7.0. Previously solubilized protein material carrying bactericidal and permeability-increasing activities was lost during dialysis against pH 7.4 buffer because of precipitation.

Citric Acid Extraction of Whole Cells (7)—Citric acid extraction of polymorphonuclear leukocytes was performed by homogenization of the cells (1 x 10^8/ml) at 0° in 0.01 M citric acid followed by incubation with shaking for 60 min. The extract was collected as the supernatant fluid after centrifugation at 25,000 x g for 30 min. The yield of bactericidal and permeability-increasing activities in the citric acid extract was approximately the same after incubation at 0° or at 37°.

Sodium Acetate Extraction of Isolated Granules (8)—The granule-rich pellet obtained by differential centrifugation as described above was extracted with approximately 10 volumes of 0.2 M sodium acetate (pH 4.0), overnight at 0° with continuous stirring. The extract was collected by centrifugation at 30,000 g x for 30 min.

Chromatographic Methods

Gel filtration chromatography was performed on Sephadex G-75 (superfine) and Sephadex G-100 (medium) at 4°. The Sephadex beads were prepared according to the instructions of the manufacturer and equilibrated in the elution buffer (see legends to Figs. 1 and 4). To increase resolution, the smallest of the superfine beads were removed by straining through a nylon net with pores of 38 μm before degassing and packing the column. Both columns were packed and eluted under a hydrostatic pressure of 15 to 25 cm of H2O maintained by a Mariotte bottle. Ion-exchange chromatography was performed on Bio-Rex 70 at 0°-4°. Details are described in the legend to Fig. 2. Fractions were eluted with a continuous gradient of increasing sodium acetate concentration produced with the aid of a gradient mixer. Salt concentrations in the eluate were determined by measuring conductivity with a four-electrode mho meter (Tetramatic-Remotron).

Concentration of Protein

Granule extracts were concentrated by ultrafiltration at 4° on Amicon Diaflo PM-10 membranes (Amicon Corp., Lexington, Mass.) before chromatography on Sephadex G-75. The active fraction obtained by ion-exchange chromatography was dialyzed against distilled water, lyophilized (without loss of biological activity), and resuspended in the elution buffer used for Sephadex G-100 chromatography.

Polyacrylamide Gel Electrophoresis

Electrophoresis in 9% polyacrylamide gels in the presence of SDS was performed according to the method of Neville (9). Dialyzed and lyophilized protein samples were resuspended in 40 μl of water containing 12.5 mM dithiothreitol and 0.75% SDS, boiled for 5 min, and applied after addition of 3 μl each of 100% sucrose and 0.1% bromphenol blue. Electrophoresis was carried out at 3 mA/gel. The gels were fixed and stained in a solution consisting of 0.25% Coomassie brilliant blue in 25.7% methanol and 9.2% glacial acetic acid for 18 h at 37°. The gels were destained by dialysis against several changes of a solution containing 5% ethanol and 5% glacial acetic acid.

Amino Acid Analysis

Analyses were performed in a Durrum D-900 amino acid analyzer.

Immunoechemical Methods

Rabbit antiserum against purified Bio-Rex fraction containing permeability-increasing and bactericidal activities was prepared by injection of 0.3 mg of protein emulsified in 1:1 mixture of distilled water/Freund's complete adjuvant (1 ml) into the foot pads. A booster injection of 0.2 mg was delivered after 3 weeks and the animals were bled 2 weeks later. Generation of antibody was determined by double immunodiffusion in 1% agarose gels according to Ouchterlony (10).

Microorganisms

E. coli strains S15 and S17 (F⁻, thi⁻, leu⁴, thr¹, lac², m.fb⁻) were kindly donated by Professor S. Nogami (Department of Chemistry, National Institute of Health, Tokyo, Japan); E. coli C600 (F+, thi¹, leu⁴, lacixe, yf¹, lacZ⁻) obtained from Dr. Robert B. Bachman (Curator, E. coli Genetic Stock Center, Department of Human Genetics, Yale University School of Medicine, New Haven, Conn.), and E. coli ML 398 225 by Dr. Milton Salton (Department of Microbiology, New York University School of Medicine, New York, N. Y.). Salmonella typhimurium MS 395 and rough mutants derived from it (R5 and R10) were kindly donated by Dr. Barbara J. Bachman (Curator, E. coli Genetic Stock Center, Department of Human Genetics, Yale University School of Medicine, New Haven, Conn.). E. coli ML 398 225 by Dr. Milton Salton (Department of Microbiology, New York University School of Medicine, New York, N. Y.). Salmonella typhimurium MS 395 and rough mutants derived from it (R5 and R10) were kindly donated by Dr. Barbara J. Bachman (Curator, E. coli Genetic Stock Center, Department of Human Genetics, Yale University School of Medicine, New Haven, Conn.).

The abbreviations used are: SDS, sodium dodecyl sulfate.

We are indebted to Dr. Edward C. Franklin and Ms. Joan Zaretzky of New York University School of Medicine who carried out these analyses.
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given by Dr. Olle Stendahl (Department of Medical Microbiology, University of Linköping, Linköping, Sweden) and other bacterial strains by Dr. Michael S. Simberloff (Department of Microbiology, Veterans Administration Hospital, New York, N. Y.). Candida albicans and Candida parapsilosis were kindly donated by Dr. Robert Lehrer (Department of Medicine, UCLA School of Medicine, Los Angeles, Calif.).

Growth in culture was either in a triethanolamine-buffered (pH 7.75 to 7.9) minimal salts medium (11) (all E. coli strains except C960) and both Candida strains), in nutrient broth (pH 7.5) (E. coli C960, S. typhimurium, Staphylococcus aureus, Bacillus subtilis, and Micrococcus lysodeikticus), or in antibiotic Medium 3 (Streptococcus faecalis). Bacterial cultures grown overnight to stationary phase were transferred to fresh medium (diluted 1:10) and the subcultures were incubated for at least 2 h at 37°C. Stationary phase cultures of Candida, obtained from 40-h incubations, were similarly subcultured and subsequently harvested after 12 to 16 h growth at 37°C. All microorganisms were harvested and used for assay of bactericidal and permeability-increasing activities during midlate logarithmic growth phase (6 to 10 x 10⁹/mL). Bacterial concentrations were determined by measuring absorbance at 550 nm with a Coleman junior spectrophotometer. The microorganisms were sedimented by centrifugation at 6000 x g for 10 min and resuspended in sterile physiological saline in the desired concentration. Bacteria grown in antibiotic Medium 3 were washed twice in 5 ml of calcium-free Krebs-Ringer phosphate buffer before resuspension in saline.

**Assays for Permeability-increasing and Microbicidal Activities**

An effect of leukocyte fractions on the permeability of Gram-negative bacteria was measured by determining the susceptibility of these bacteria to actinomycin D. The use of actinomycin D for assay of envelope permeability is based on the following observations. 1) Most Gram-negative bacterial envelopes are impermeable to actinomycin D (12). Hence, this antibiotic normally does not affect synthesis of RNA and protein by Gram-negative bacteria. 2) Bacterial concentrations of purified fractions of polymorphonuclear leukocytes produce little or no inhibition of bacterial macromolecular synthesis for at least 2 h (1, 2, 4). Therefore, inhibition of RNA and protein synthesis by actinomycin D is dose-dependent, the permeability-increasing activity can be quantitatively determined, using the following equation:

\[ \text{Activity} = \frac{\text{bacterial } [%^{14}C] \text{leucine incorporation} - \text{leukocyte fraction } + \text{actinomycin D}}{ \text{leukocyte fraction} - \text{actinomycin D} } \times 100. \]

One arbitrary unit of permeability-increasing activity is defined as a 50% reduction by actinomycin D of [%¹⁴C]leucine incorporation by 2.5 x 10⁹ bacteria.

**Assay for Microbicidal Activity**

After 30-min incubation, 10-µl samples were taken from the incubation mixtures, diluted in trichloroacetic acid, precipitated with cold trichloroacetic acid, and assayed for incorporation into bacterial protein in the presence of actinomycin D (see "Experimental Procedures"). The permeability effect of a given polymorphonuclear leukocyte fraction is measured by calculating the inhibition of bacterial synthesis specifically attributable to actinomycin D. Because the envelope effect of our purified bactericidal protein fraction that renders Gram-negative bacterial protein synthesis susceptible to inhibition by actinomycin D is dose-dependent, the permeability-increasing activity can be quantitatively determined, using the following equation:

\[ \text{Activity} = \frac{\text{bacterial } [%^{14}C] \text{leucine incorporation} - \text{leukocyte fraction} - \text{actinomycin D}}{ \text{leukocyte fraction} - \text{actinomycin D} } \times 100. \]

One arbitrary unit of permeability-increasing activity is defined as a 50% reduction by actinomycin D of [%¹⁴C]leucine incorporation by 2.5 x 10⁹ bacteria.

**Other Assays**

Protein was determined by the method of Lowry et al. (14) using bovine serum albumin as standard. Phospholipase A₂ was assayed by using autolysed [¹⁴C]cholesterol-labeled E. coli as substrate (15). Esterase activity was assayed by using the synthetic substrates benzoyl-L-tyrosine ethyl ester and 4-nitro-L-arginine methyl ester (16). Myeloperoxidase, collagenase, and elastase content of purified leukocyte fractions was determined by the single radial immunodiffusion method of Mancini (17).

**RESULTS**

**Preliminary Findings; Extraction of Permeability-increasing Activity**

An important characteristic of the highly purified bacterial protein fraction from rabbit polymorphonuclear leukocytes is that its bactericidal effect is closely linked to an almost immediate increase in the envelope permeability of susceptible bacteria (1, 3). To identify the possible existence in preparations of human polymorphonuclear leukocytes of a similar antimicrobial activity, we therefore initially used our assay for permeability-increasing activity. To extract biologically active cationic proteins from leukocytes, different extraction procedures have been used by different investigators (1, 7, 8, 18). In Table I the use of 0.16 M sulfuric acid (2, 18), 0.2 M sodium acetate, pH 4.0 (8), and 0.01 M citric acid (7) for extraction of permeability-increasing activity from human leukocytes is compared. As we found to be the case for rabbit leukocytes (1), 0.16 M sulfuric acid treatment of whole cell homogenates, prepared in water, is the most effective means of extracting permeability-increasing activity (and bactericidal activity as well, not shown here). Cell populations consisting of 90% mononuclear cells contain 70 to 75% less permeability-increasing activity than populations consisting of 90% polymorphonuclear cells. Thus, permeability-increasing activity is extracted mainly from the polymorphonuclear leukocytes. Again similar to our experience with rabbit leukocytes.

**Table I**

Comparison of various procedures for extraction of permeability-increasing activity from human leukocytes

Leukocyte populations were fractionated as described under "Experimental Procedures." Polymorphonuclear cell populations contained <10% mononuclear cells and mononuclear cell populations <10% polymorphonuclear cells, as judged by differential cell counts. The three extraction procedures and the measurement of permeability-increasing activity are described under "Experimental Procedures." Units are given as mean ± S.E. of (n) extractions. One unit is the amount of permeability-increasing protein that causes a 50% reduction of incorporation of [%¹⁴C]leucine into bacterial protein in the presence of actinomycin D (see "Experimental Procedures").

<table>
<thead>
<tr>
<th>Normal</th>
<th>Chronic myelocytic leukemia (polymorphonuclear)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction</td>
<td></td>
</tr>
<tr>
<td>Polymorphonuclear</td>
<td>Mononuclear</td>
</tr>
<tr>
<td>units/2.5 x 10⁸ cells</td>
<td></td>
</tr>
<tr>
<td>0.16 M H₂SO₄/whole cell homogenate</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>0.2 M acetate (pH 4.0)/granule-rich pellet</td>
<td>8.8 ± 0.8</td>
</tr>
<tr>
<td>0.01 M citric acid/whole cell homogenate</td>
<td>2.8</td>
</tr>
</tbody>
</table>
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Leukocytes, sulfuric acid extracts of isolated granules contained less than 30% of the permeability-increasing activity recovered from whole homogenates (not shown), i.e., generally less than can be extracted from granules with 0.2 M sodium acetate. Citric acid treatment does not effectively solubilize permeability-increasing (and bactericidal) activity.

Table I also shows that both sulfuric acid and sodium acetate extracts of polymorphonuclear leukocytes from normal individuals and from patients with chronic myelocytic leukemia contain comparable permeability-increasing (and bactericidal) activity. We therefore made use of the large numbers of polymorphonuclear leukocytes available from patients with chronic myelocytic leukemia to prepare extracts for further purification.

A serious drawback of sulfuric acid extracts of human leukocytes is that such extracts when concentrated by ultrafiltration before column chromatography, undergo precipitate formation with large loss of biological activity. This does not happen with acetate extracts. Hence extracts prepared with sodium acetate were used for further purification.

Purification

Gel Filtration Chromatography on Sephadex G-75—Chromatography of the concentrated granule extract on Sephadex G-75 (Fig. 1) produces several protein peaks. Practically all permeability-increasing activity is eluted as a single peak (Fractions 35 to 39) corresponding to a discrete protein peak (5 to 6% of the total protein applied) just after the void volume. Nearly all of the granule extract’s bactericidal activity towards E. coli is co-eluted with the permeability-increasing activity; less than 4 μg of each fraction that contains permeability-increasing activity produces greater than 90% killing of 5 × 10⁷ E. coli. In contrast, 30 to 340 μg of the other protein peak fractions, tested at neutral pH, have no detectable bactericidal effect.

Ion Exchange Chromatography on Bio-Rex 70—The Sephadex G-75 fractions containing permeability-increasing and bactericidal activities towards E. coli (Fractions 35 to 39) were subjected to further chromatography on an ion exchange resin (Bio-Rex 70) using a continuous gradient of sodium acetate (0.1 to 1.5 M) for elution. Both activities bind to the resin and are co-eluted in a single discrete protein peak with approximately 0.75 M sodium acetate (Fig. 2). The specific activity of the fractions across this peak is essentially constant and less than 3 μg of protein in each fraction produces greater than 90% killing of 5 × 10⁷ E. coli. The three other protein peaks that are eluted with lower salt concentrations contain no detectable bactericidal activity and less than 1% of the total permeability-increasing activity applied.

Isoelectric Focusing—Isoelectric focusing of the active fraction obtained by Bio-Rex chromatography is shown in Fig. 3. One major protein peak with an apparent isoelectric point of pH 9.8 is recovered that contains 98 to 99% of the protein and all of the permeability-increasing and bactericidal activities. There is no enrichment of other biological activity; in fact, the peak fractions have lower specific activities probably because of partial isoelectric precipitation.

Molecular Weight Determination—Chromatography of the active Bio-Rex fraction on Sephadex G-100 is shown in Fig. 4. A single symmetrical protein peak is eluted with an apparent molecular weight of 60,000. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of this fraction also reveals a single major protein of approximately 58,000 (Fig. 4). A second (trace) component occurs with slightly greater mobility. The minor component with pI 8.5 isolated by isoelectric focusing has the same mobility in sodium dodecyl sulfate-polyacrylamide electrophoresis. This material is devoid of permeability increasing or bactericidal activity indicating that this trace...
The amino acid composition of the purified bactericidal/permeability-increasing protein is shown in Table III. The overall composition is very similar to that of the bactericidal/permeability-increasing protein from rabbit leukocytes. The acidic amino acid residues outnumber the basic ones, suggesting that acidic amino acids exist in the amide form, thus contributing to the basic character of these proteins (8).

**Permeability-increasing and Bactericidal Activities of Purified Fraction**

Concentration Dependence – Fig. 6 shows that the purified (Bio-Rex) fraction produces in *E. coli* a dose-dependent (over a range from 1 to 4 μg) loss of viability. These bactericidal concentrations of the protein also produce a dose-dependent inhibition of [14C]leucine incorporation into trichloroacetic acid-precipitable material in the presence of actinomycin D (but only slight inhibition in the absence of the antibiotic; see "Experimental Procedures"). Quantitative differences between the effects on viability and permeability produced by a

**Table II**

**Purification of permeability-increasing and bactericidal activities from human polymorphonuclear leukocytes**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein</th>
<th>Permeability-increasing activity</th>
<th>Bactericidal activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 4.6 extract of granules</td>
<td>264 (100)</td>
<td>600 (100)</td>
<td>2.3</td>
</tr>
<tr>
<td>Sephadex G-75 chromatography</td>
<td>14.4 (5.4)</td>
<td>645 (107)</td>
<td>45</td>
</tr>
<tr>
<td>Bio-Rex chromatography</td>
<td>4.6 (1.5)</td>
<td>356 (59)</td>
<td>89</td>
</tr>
</tbody>
</table>

*The micrograms of protein required to kill more than 90% of 5 x 10⁶ *E. coli.*
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**pH Dependence**—Both permeability-increasing and bactericidal activities are maximal at pH 7.0 (Fig. 7).

**Effect of Heat Treatment**—Heating at 60°C for 10 min in distilled H₂O has little effect on permeability-increasing and

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**Table III**

Amino acid composition of purified bactericidal/permeability-increasing protein from human polymorphonuclear leukocytes

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>8.5</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.2</td>
</tr>
<tr>
<td>Serine</td>
<td>7.8</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>9.1</td>
</tr>
<tr>
<td>Proline</td>
<td>7.9</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.9</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.5</td>
</tr>
<tr>
<td>Half cystine</td>
<td>N.D.</td>
</tr>
<tr>
<td>Valine</td>
<td>7.9</td>
</tr>
<tr>
<td>Methionine</td>
<td>3.3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.5</td>
</tr>
<tr>
<td>Leucine</td>
<td>10.6</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.3</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.8</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.6</td>
</tr>
<tr>
<td>Lysine</td>
<td>7.9</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* N.D. = not detected.

given amount of purified protein is probably attributable to the use of two different assays.

**Effects on Viability and Permeability Can Be Produced Without Evidence of Net Macromolecular Degradation**—The bactericidal and permeability-increasing effects of the purified protein on *E. coli* S15 are produced without demonstrable breakdown of bacterial protein and RNA, but are accompanied by a small net hydrolysis of bacterial phospholipids (Table IV). Use of a phospholipase A-deficient mutant (*E. coli* S17) (23) shows that this net degradation is the result of activation of the bacterial phospholipases. These results also show that this activation is not required for expression of the effects on viability and permeability.

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**Table IV**

Bactericidal and permeability-increasing effects of purified protein on *E. coli* without net macromolecular degradation

*E. coli* S15 or S17 (5 x 10⁸ bacteria) were incubated with 4 µg of Bio-Rex fraction (Fig. 2) in the standard incubation mixture for 30 min at 37°C. Bactericidal and permeability-increasing (inhibition of [³H]leucine incorporation) activities were determined as described under “Experimental Procedures.” Values are expressed as per cent of values obtained with *E. coli* incubated alone. To measure degradation of bacterial protein, RNA, and phospholipids, these constituents were labeled during growth of the bacteria with, respectively, [¹⁴C]leucine, [³H]uracil, and [¹⁴C]oleic acid, exactly as previously described (3, 20). Loss of acid-precipitable radioactivity, expressed as per cent of values obtained in *E. coli* incubated alone, was used as a measure of degradation of radiolabeled protein and RNA (20). Accumulation of [¹⁴C]-labeled fatty acid and [³H]-labeled lysocompounds, measured by thin layer chromatography of lipid extracts, and expressed as per cent of total bacterial lipid radioactivity, provided an index of net hydrolysis of phospholipids. The results shown are the means of at least three individual determinations of bactericidal and permeability-increasing activity, and of phospholipid hydrolysis. Release of acid-soluble radioactivity from labeled protein and RNA was determined in duplicate in one of these three experiments.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Loss of viability</th>
<th>[³H]Leucine incorporation</th>
<th>Degradation of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>% -ActD +ActD</td>
<td>Protein</td>
</tr>
<tr>
<td><em>E. coli</em> S15</td>
<td>99.8</td>
<td>91</td>
<td>25</td>
</tr>
<tr>
<td><em>E. coli</em> S17</td>
<td>97.9</td>
<td>105</td>
<td>22</td>
</tr>
</tbody>
</table>

* Act D, actinomycin D.
bactericidal activities but exposure to 80° markedly reduces both activities (Fig. 8). After boiling both activities are lost (not shown). In dilute acetic acid (pH 3.5) the two activities are slightly more resistant to heat treatment but are largely inactivated at 95°.

**Effect of Divalent Cations and Heparin**—Low concentrations of Ca\(^{++}\) or Mg\(^{++}\) and heparin sulfate inhibit the permeability and bactericidal effects of the purified protein (Table V). One hundred fifty millimolar concentrations of Na\(^{+}\) or K\(^{+}\) have no inhibitory effect on either activity (not shown).

**Biological Activities Toward Various Microorganisms**—Table VI compares the sensitivities of a range of Gram-

![Fig. 7. Effect of pH on permeability-increasing and bactericidal activities of purified protein. Permeability-increasing activity was determined by incubation of 5 \times 10^7 E. coli S15 with 4 pg of protein (Bio-Rex fraction) in the presence (□) and absence (●) of actinomycin D in the standard incubation mixture as described under “Experimental Procedures” except that different buffers of indicated pH were used. Bactericidal activity was determined by incubating 5 \times 10^7 E. coli with 2 pg of protein in the same incubation mixtures. The buffers used were: sodium acetate, pH 4.0 to 6.0; sodium phosphate, pH 6.5; Tris/HCl, pH 7.0 to 9.0. All buffer concentrations were 25 mM. Permeability-increasing activity could not be determined below pH 6.5 because at low pH, \(^{14}C\)leucine incorporation by E. coli incubated alone is markedly depressed. Results are expressed as per cent of E. coli incubated alone at the indicated pH and represent the mean of two closely similar experiments carried out in duplicate.

![Fig. 8. Effect of heat treatment on (A) permeability-increasing and (B) bactericidal activities of purified protein. Sixty micrograms of protein (Bio-Rex fraction) in 150 μl of distilled water were preincubated for 10 min at 20° (circles), 60° (squares) or 80° (triangles). After preincubation, aliquots of the treated protein, at the indicated concentration, were incubated with 5 \times 10^7 E. coli S15 in the standard incubation mixture in the presence (open symbols) and absence (closed symbols) of actinomycin D. Bactericidal and permeability-increasing activities were determined as described under “Experimental Procedures.” Results are expressed as per cent of E. coli incubated alone and represent the mean of two closely similar experiments.

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**Table V**

Inhibition of permeability-increasing and bactericidal activities of purified fraction by Mg\(^{++}\), Ca\(^{++}\), and heparin

<table>
<thead>
<tr>
<th>Incubation</th>
<th>(^{14}C)Leucine incorporation(^a)</th>
<th>Colony-forming units</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli alone</td>
<td>100</td>
<td>97</td>
<td>9.9 \times 10^7</td>
</tr>
<tr>
<td>E. coli + Bio-Rex fraction</td>
<td>86</td>
<td>21</td>
<td>6.6 \times 10^7</td>
</tr>
<tr>
<td>E. coli + Bio-Rex fraction + 2.5 mM Mg(^{++})</td>
<td>101</td>
<td>85</td>
<td>6.1 \times 10^7</td>
</tr>
<tr>
<td>E. coli + Bio-Rex fraction + 2.5 mM Ca(^{++})</td>
<td>95</td>
<td>95</td>
<td>1.2 \times 10^4</td>
</tr>
<tr>
<td>E. coli + Bio-Rex fraction + 5 μg heparin</td>
<td>101</td>
<td>92</td>
<td>6.2 \times 10^7</td>
</tr>
</tbody>
</table>

\(^a\) Values are expressed as per cent of values obtained with E. coli incubated alone.

\(^b\) Act D, actinomycin D.
TABLE VI
Susceptibility of different microorganisms to permeability-increasing and microbicidal effects of purified human polymorphonuclear leukocyte fractions

Incubations and assays were carried out as described under "Experimental Procedures." The number of microorganisms added per incubation mixture was 5 x 10⁴ for each species. The designations one to four + indicate the amount of purified protein, either from Bio-Rex (Fig. 2) or from Sephadex G-100 (Fig. 4) chromatography, necessary to produce >90% effect on microbial viability and permeability, measured as detailed under "Experimental Procedures": +++++, >1 mg; ++++, 1 to 2.5 mg; ++, 2.5 to 5 mg; +, 5 to 10 mg; 0, no detectable effect with >20 mg; N.T., not tested; r, a microorganism natively susceptible to actinomycin D. All experiments, except those with B. subtilis and M. lysodeikticus, were carried out at least twice in duplicate. Bacterial chemotype or colony morphology are indicated in parentheses: r, rough; s, smooth.

<table>
<thead>
<tr>
<th>Gram-negative</th>
<th>Bacteria</th>
<th>Permeability</th>
<th>Loss of viability</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>S15 (r)</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>S17 (r)</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>C600 (r)</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>H0129 (s)</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>ML 308 225 (s)</td>
<td>N.T.</td>
<td>++</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>G-30 (Rc)</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>G-30 + galactose (s)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>R10-395 (Rd)</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>R5-395 (Rb)</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td>MS-395 (s)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

| Gram-positive | *Staphylococcus aureus* | Qui | 0 |
|               | W46 | 0 |
|               | C1 | 0 |
|               | Ferrari (coagulase +) | 0 |

| Streptococcus faecalis | BS9 | 0 |
| B. subtilis | 0 |
| M. lysodeikticus | 0 |
| Fungi | Candida albicans | 0 |
| Candida parapsilosis | 0 |

**DISCUSSION**

The bactericidal protein isolated in nearly pure form from human polymorphonuclear leukocytes granules differs from other human leukocyte antimicrobial proteins, that have previously been identified and purified (8, 21), in a number of its molecular and biological properties including molecular weight, amino acid composition, as well as bactericidal potency and specificity. In contrast, in all these properties this protein from human leukocytes closely resembles the purified bactericidal permeability-increasing protein from rabbit polymorphonuclear leukocytes (2). The bactericidal proteins from the two species have molecular weights between 50,000 and 60,000, very similar amino acid composition and isoelectric points greater than 9.5. Another common feature of these proteins is that they are maximally active at neutral pH. Both proteins exhibit potent bactericidal activity towards several strains of *E. coli* and *S. typhimurium* (concentrations as low as 10 nm of either protein kill more than 90% of 10⁴ bacteria/mil) but no apparent activity towards several Gram-positive bacterial species.

The bactericidal activity of the purified protein is closely linked to an effect on the envelopes of susceptible bacteria that is recognizable as an almost immediate breakdown of the permeability barrier to actinomycin D. As has been shown in detail in our studies of the structural and functional effects of purified rabbit leukocyte fractions (3, 22) the increase in envelope permeability reflects only limited structural alterations and is not part of a general disorganization of the bacteria. In fact, although loss of ability to multiply and increased permeability are manifest within minutes after exposure of the bacteria to either the human or the rabbit bactericidal protein, the bacteria exhibit little or no macromolecular degradation and remain capable of almost unimpaired macromolecular biosynthesis for incubation periods of up to 2 h (8; Table IV). Both the rabbit and the human bactericidal/permeability-increasing proteins initiate net phospholipid hydrolysis upon contact by activating the bacterial phospholipases (23; Table IV).

One difference between the human and rabbit bactericidal/permeability-increasing proteins is the close connection of a phospholipase A₂ with the rabbit protein during extensive purification (1, 23). This phospholipase A₂ attacks the phospholipids of intact *E. coli*, but only when the enzyme is associated with the bactericidal/permeability-increasing protein (2, 23). In contrast, although human polymorphonuclear leukocyte granules also contain phospholipase A₂ activity (be it at levels that are less than 5% of those in rabbit preparations), we have never observed phospholipase A₂ activity in association with the human bactericidal/permeability-increasing fractions.

The cationic bactericidal/permeability-increasing protein is at least 20 to 50 fold more active towards all rough (and a number of smooth) strains of *E. coli* and *S. typhimurium* than any other protein fraction, derived from human leukocyte granules, so far examined (Fig. 1; Ref. 21). What the contribution of this exceedingly potent bactericidal protein to the overall antimicrobial activity of polymorphonuclear leukocyte constituents is not known. In a recent in vitro study the bactericidal activity of the myeloperoxidase/H₂O₂/halide system towards *E. coli* was compared with that of other cationic granule constituents, by determining the activity of citric acid extracts of human polymorphonuclear leukocyte granules at pH 5.0 in the presence and absence of H₂O₂ and chloride (7). Because without supplementation with H₂O₂ and chloride the extract manifested little bactericidal activity it was suggested that relative to the myeloperoxidase-mediated antimicrobial system other cationic antimicrobial proteins are not particularly effective. As shown in Table I, citric acid extraction of granules yields only 10 to 15% of the bactericidal/permeability-increasing activity that can be extracted with stronger acids. Furthermore, at pH 5.0 the bactericidal/permeability-increasing protein is almost inactive (Fig. 7). In future comparative studies one must therefore take into consideration the specific physical and biological properties of the various antimicrobial proteins and systems of the leukocyte. In addition, it is possible that these various systems, although they probably do act in concert, exert their maximal effects on bacteria under dissimilar conditions. In this regard the role of the intravacuolar pH may be an important one. Thus, antimicrobial proteins, such as the bactericidal/permeability-increasing protein that are maximally active at neutral pH (unlike the myeloperoxidase system) may fulfill their function very early.

*Manuscript in preparation.*
after intracellular sequestration of bacteria, or even concomi-
tant with vacuole formation and degranulation, before a pH
drop has occurred (24).

The fact that a number of gram negative bacterial species
are exquisitely sensitive to the bactericidal/permeability-in-
creasing protein, but that all Gram-positive bacteria so far
examined and two strains of the fungus Con&da are highly
resistant, correlates with evidence that binding is necessary
for expression of biological activity. Thus, whereas all sensi-
tive Gram-negative microorganisms show a direct correlation
between the extent of binding of either human or rabbit
bactericidal/permeability-increasing protein and the degree of
susceptibility to their bactericidal as well as permeability-
increasing effects, resistant Gram-negative and -positive bac-
teria show little or no binding (1). This correlation extends
further to the length of the polysaccharide chain of the outer
membrane lipopolysaccharides1 (Table VI). This and other
findings (2) provide support for our concept that the primary
interaction of the cationic bactericidal/permeability-increas-
ing protein of both human and rabbit leukocytes is with
surface structures, perhaps the negatively charged lipopoly-
saccharides, of susceptible Gram-negative bacteria. Such in-
teraction might expose previously covered hydrophobic re-
gions, thereby permitting passage of actinomycin D through
the normally impermeable outer membrane (25, 26). What the
connection is between this completely reversible alteration in
envelope properties to the irreversible loss of ability to multi-
ply1 (3), remains to be determined.

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REFERENCES

1. Weiss, J., Franson, R. C., Beckerdite, S., Schmeidler, K., and
2. Weiss, J., Elsbach, P., Franson, R. C., Beckerdite-Quagliata,
Abstr.
89
Press, New York
515
S. A. 51, 877–883
13. Beckerdite, S., Mooney, C., Weiss, J., Franson, R., and Elsbach,
(1951) J. Biol. Chem. 193, 265–275
15, 386–388
24, 427–441
Immunochemistry 2, 235–254
19. Odeberg, H., Olofsson, T., and Olsson, I. (1975) Brit. J. Haema-
tol. 29, 427–441
Bacteriol. 115, 900–907
Infer Immunol. 15, 556–559
23–33
Purification and characterization of a potent bactericidal and membrane active protein from the granules of human polymorphonuclear leukocytes.

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