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, dis-
Bio-Rex 70 (200 to 400 mesh) was obtained from Bio-Rad Laboratories. Ammonium mixtures with pH range of 7 to 9 and 9 to 11 (Ampholine 1809-136 and 1809-146) were obtained from LKB-Produkter AB. Acrylamide and N,N'-methylenebisacrylamide were obtained from Eastman Kodak Co. and sodium dodecyl sulfate (sodium lauryl sulfate) from Pierce Chemical Co. Sucrose (ultrapure, density gradient grade) was acquired from Schwarz/Mann and agarose (industre Ab) from L’Industrie Biologique Francaise S.A. Proteins used as standards for gel filtration and gel disc electrophoresis were bovine serum albumin (Miles Laboratories, Inc.), ovalbumin and chymotrypsinogen A (Pharmacia Fine Chemicals), and egg lysozyme (Sigma Co.). Dextran (clinical grade, average molecular weight 73,200) was from Sigma. Ficoll 400 was obtained from Pharmacia Fine Chemicals and Isopaque from Winthrop Laboratories. Bovine serum albumin (Miles Laboratories, Inc.), ovalbumin and chymotrypsinogen A (Pharmacia Fine Chemicals), and egg lysozyme were used as standards for gel filtration and gel disc electrophoresis were obtained from Difco Laboratories. Hanks’ balanced salt solution (without phenol red) was supplied by Microbiological Associates and actinomycin D by Merck, Sharp and Dohme. L-[l-14C]Leucine (48 Ci/mol) was obtained from International Chemical and Nuclear Corporation. Polyacrylamide gel electrophoresis was performed on Sehaphex 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 2). To increase resolution, the smallest of the superfine beads were removed by draining 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.

Chromatographic Methods

Gel filtration chromatography was performed on Bio-Rex 70 at 0-4°C. 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-Kemtron).

Concentration of Protein

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

Sodium Acetate Extraction of Isolated Granules

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°C with continuous stirring. The extract was collected by centrifugation at 30,000 × g for 30 min.

Preparation of Granule-rich Fraction

To obtain granules the leukocytes were resuspended in 0.34 M sucrose (2 × 106 cells/ml) and homogenized at 0°C in a Potter-Elvehjem glass homogenizer equipped with a motor-driven Teflon pestle. A mononuclear cell fraction was obtained by centrifugation at 400 × g for 10 min. The supernatant was recentrifuged at 2,500 × g for 10 min, yielding the mononuclear cell fraction. These cells were washed twice with Krebs-Ringer phosphate before homogenization.

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

Amino Acid Analysis

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

Microorganisms

E. coli strains S15 and S17 (F⁻, thi⁻, leu⁻, thr⁻, lac⁺, mel⁻) were kindly donated by Professor S. Nojima (Department of Chemistry, National Institute of Health, Tokyo, Japan). E. coli C600 (F⁺, thi⁻, thr⁻, leu⁺, lacY¹, tonA21, supE44, A⁻, lacY¹, lacZ⁺) was provided by Dr. Barbara J. Bachman (Curator, E. coli Genetic Stock Center, Department of Human Genetics, Yale University School of Medicine, New Haven, Conn.), and E. coli ML 308 225 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 obtained by Dr. Robert J. Zaretzky of New York University School of Medicine who carried out these analyses.

The abbreviations used are: SDS, sodium dodecyl sulfate.

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Permeability-increasing activities was lost during dialysis against pH 7.4 buffer because of precipitation. Previously solubilized protein material carrying bactericidal activity was dialyzed against 2 mM Tris/HCl, pH 7.0. Neutralization of the extract by prolonged dialysis increased resolution, the smallest of the superfine beads were removed by draining 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.

Chromatographic Methods

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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 60% sucrose and 0.1% bromphenol blue. The gels were fixed and stained in a solution consisting of 0.25% Coomassie brilliant blue in 25.7% ethanol and 9.2% glacial acetic acid for 18 h at 37°C. The gels were destained by dialysis against several changes of a solution containing 5% ethanol and 5% glacial acetic acid.

Analysis of leucocytes was performed in a Durrum D-900 amino acid analyzer.

Immunological 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 a 1:1 mixture of distilled water/Freund's complete adjuvant (1 ml) into the footpad. 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).

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Bactericidal Protein from Human Leukocytes

given by Dr. Olle Stendahl (Department of Medical Microbiology, University of Linköping, Linköping, Sweden) and other bacterial strains by Dr. Michael S. Simberkoff (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 C600) or in nutrient broth (pH 7.6) (E. coli C600, 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 3 to 4 h at 37°. Stationary phase cultures of Candida, obtained from 40-h incubations, were similarly subcultured and subsequently harvested after 13 to 16 h growth at 37°.

All microorganisms were harvested and used for assay of bactericidal and permeability-increasing activities during midlate logarithmic growth phase (6 to 10 x 10^9/ml). Microbial 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**

Typical incubation mixtures contained from 5 x 10^6 to 8 x 10^7 microorganisms in a total volume of 0.4 ml of sterile physiological saline that also contained 10 μmol of Tris/HCl buffer (pH 7.0), 25 μl of Hanks' solution (without phenol red), 250 μg of vitamin-free casein, and leukocyte protein fraction in the indicated amount. Incubations were carried out for 30 min at 37°.

**Assay for Increased Microbial Envelope Permeability**

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 membranes of 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) Bactericidal 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 heated leukocyte (D plus a polymorphonuclear leukocyte fraction reflects an increase in microbial envelope permeability to the antibiotic. Assays of permeability-increasing activity were carried out in the incubation mixture described above by determining the bacterial incorporation of [1-14C]leucine (0.063 μCi; 0.15 μl) into cold trichloroacetic acid-precipitable material in the presence or absence of actinomycin D (12.5 μg) (1, 13). The same results are obtained using [1-14C]urocanic acid as precursor (13). 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:

\[
\text{Permeability-increasing activity} = \frac{\text{bacterial} \ [14C] \text{leucine incorporation}}{\text{bacterial} \ [14C] \text{leucine incorporation}} \times 100
\]

One arbitrary unit of permeability-increasing activity is defined as a 50% reduction by actinomycin D of [1-14C]leucine incorporation by 2.5 x 10^9 bacteria.

**Assay for Microbicidal Activity**

After 30-min incubation, 10-μl samples were taken from the incubation mixtures, diluted twice in cold trichloroacetic acid, and plated on either nutrient agar (E. coli, S. typhimurium, S. subtilis), brain heart infusion agar (S. aureus, M. lysodeikticus) or antibiotic Medium 3 agar (all strains). Sensitivity to the microbicidal activity of the purified fraction obtained in this study was not affected by the agar medium used for colony formation. The number of colony-forming units on the plates was determined after incubation at 37° overnight (all bacterial strains except M. lysodeikticus), 48 h (Candida), or 2 to 3 days at room temperature (M. lysodeikticus).

**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 [1-14C]oleate-labeled E. coli as substrate (15). Esterase activity was assayed by using the synthetic substrates benzoyl-L-tyrosine ethyl ester and p-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 bactericidal 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 1 the use of 0.16 N 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 N 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 1**

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 [1-14C]leucine into bacterial protein in the presence of actinomycin D (see "Experimental Procedures").

<table>
<thead>
<tr>
<th>Extraction</th>
<th>Normal</th>
<th>Chronic myelocytic leukemia (polymorphonuclear)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymorphonuclear</td>
<td>Mononuclear</td>
<td></td>
</tr>
<tr>
<td>units/2.5 x 10^9 cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.16 N H_2SO_4/whole cell homogenate</td>
<td>22 ± 3</td>
<td>6.0 ± 2.4</td>
</tr>
<tr>
<td>0.2 M acetic acid (pH 4.0)/granule-rich pellet</td>
<td>8.8 ± 0.8</td>
<td>8.4 ± 3.2</td>
</tr>
<tr>
<td>0.01 M citric acid/whole cell homogenate</td>
<td>2.8</td>
<td>(3)</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 (19)) 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 precipitation 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 toward 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 x 10⁸ 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 x 10⁸ 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 either 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 component is not related to the bactericidal effect.

Fig. 1. Sephadex G-75 chromatography of acid extract of human polymorphonuclear leukocyte granules. Acetate (pH 4.0) extract of human leukocyte granules (264 mg of protein) was applied in 3.3 ml of column buffer to a Sephadex G-75 (superfine) column (9.5 x 90 cm) equilibrated in 0.2 M sodium acetate, pH 4.0. Elution with gel buffer was carried out at a constant flow rate of 5 ml/h. Fractions of 4.7 ml were collected and assayed for absorbance at 280 nm. Bactericidal and permeability-increasing activities towards E. coli S15 were determined as described under "Experimental Procedures." Less than 4 µg of the protein recovered in the second peak, which contained all the recovered permeability-increasing (PI) activity (□—□—□), was sufficient to kill more than 90% of 5 x 10⁸ E. coli S15. None of the other fractions had any bactericidal effects when tested in the amounts indicated in parentheses above the peaks. The symbol > refers to the untested possibility that even greater amounts might have been bactericidal.

Fig. 2. Bio-Rex 70 chromatography of pooled bactericidal/permeability-increasing (PI) fractions (Fractions 35 to 39) obtained by Sephadex G-75 chromatography. The pooled fractions (14.4 mg of protein) previously dialyzed against 0.1 M sodium acetate containing 0.05 M Tris/HCl buffer, pH 7.4, were applied to a Bio-Rex 70 column (1.25 x 25 cm) equilibrated with the same buffer. Elution was carried out first with 2 to 3 column volumes of this buffer followed by a 400-ml linear gradient of buffered sodium acetate (0.1 to 1.5 M) and, finally, 2 column volumes of buffered 1.5 M sodium acetate. Fractions of 8.4 ml were collected at a flow rate of 11.3 ml/h and assayed for absorbance at 280 nm. Bactericidal and permeability-increasing activities were determined as described in the legend to Fig. 1. Sodium acetate concentrations were determined by conductivity as described under "Experimental Procedures." Protein recovery was >95%.
and chronic myelocytic leukemic leukocytes (Fig. 5), indicating that mature polymorphonuclear leukocytes from normal individuals and from patients with chronic myelocytic leukemia contain an immunologically identical bactericidal protein.

Immunological Identity of Bactericidal Protein from Normal and Chronic Myelocytic Leukemia Extracts

Antibody raised in sera of two New Zealand White rabbits challenged with the active Bio-Rex fraction produces an identical immunoprecipitation reaction with extracts of normal and chronic myelocytic leukemic leukocytes (Fig. 5), indicating that mature polymorphonuclear leukocytes from normal individuals and from patients with chronic myelocytic leukemia contain an immunologically identical bactericidal protein.

Amino Acid Composition

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).

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]lysine 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 45,000- to 50,000-dalton protein contributes neither biological activity to the purified fraction.

Permeability-increasing and Bactericidal Activities of Purified Fraction

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]lysine 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 45,000- to 50,000-dalton protein contributes neither biological activity to the purified fraction.

Summary of Purification—The purification procedure is summarized in Table II. Isoelectric focusing or gel filtration chromatography of the Bio-Rex fraction did not yield any further enrichment of either the permeability-increasing or bactericidal activities. Single radial immunodiffusion analysis reveals contamination with myeloperoxidase of less than 0.5% and no detectable contamination with either collagenase or elastase. The active fraction shows neither neutral esterase nor phospholipase A₂ activity.

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Bactericidal Protein from Human Leukocytes

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

Fig. 5. Double immunodiffusion of normal and chronic myelocytic leukemia polymorphonuclear leukocyte granule extracts. Rabbit antiserum generated against bactericidal/permeability-increasing fraction obtained by Bio-Rex chromatography was added to the center well and the two extracts were added to alternating peripheral wells (48 μg of protein of extract of leukemic cells; 45 μg of protein of extract of normal cells).

Table III

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

Lyophilized protein samples acid hydrolyzed (6 N HCl), 20 h at 100°C. Bactericidal/permeability-increasing protein was obtained by Sephadex G-100 chromatography (Fig. 4).

<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>Throneine</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>Glycline</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.

Fig. 6. Effect of increasing concentrations of purified (Bio-Rex) protein on sensitivity of E. coli S15 to actinomycin D (---) and on bacterial viability (-----). E. coli, 5 x 10⁹ was incubated with increasing concentrations of Bio-Rex fraction in the presence (O) and absence (O) of actinomycin D as described under "Experimental Procedures." [¹⁴C]Leucine incorporation by E. coli into trichloroacetic acid-precipitable material is expressed as per cent of E. coli incubated alone for 30 min (>2000 cpm; 1 to 2 nmol). Bacterial viability is also expressed as per cent of E. coli incubated alone and is determined for all samples in the absence of actinomycin D. Results shown represent the mean of four closely similar experiments.

Table IV

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

E. coli S15 or S17 (5 x 10⁹ 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 [¹⁴C]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, [¹⁴C]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 radioabeled protein and RNA (20). Accumulation of [¹⁴C]-labeled fatty acid and [¹⁴C]-labeled lyocompounds, 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>[¹⁴C]Leucine incorporation</th>
<th>Degradation of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ActD&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+ActD&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>E. coli S15</td>
<td>99.8</td>
<td>91</td>
<td>25</td>
</tr>
<tr>
<td>E. coli S17</td>
<td>97.9</td>
<td>105</td>
<td>22</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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 Ca2+ or Mg2+ 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-

negative and Gram-positive bacterial species to the biological activities of the purified protein. Two main findings tenta-

tively define the antimicrobial specificity of this bactericidal
agent. 1) Whereas concentrations as low as 1 μg/ml produce greater than 90% killing of several strains of Gram-negative E. coli and S. typhimurium, concentrations as high as 160 μg/ml do not affect the viability of any of four species of Gram-

positive bacteria, and of two species of Candida, tested either
at pH 7.0 or at pH 5.5. 2) Of the Gram-negative bacterial
species tested, rough strains with defective lipopolysaccha-
rides are more sensitive to the permeability and bactericidal
effects of the purified fraction than are the corresponding
smooth strains.

In all susceptible Gram-negative microorganisms, loss of
viability is accompanied by an increase in permeability.

---

**Table V**

<table>
<thead>
<tr>
<th>Incubation</th>
<th>[14C]Leucine incorporation</th>
<th>Colony-forming units</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli alone</td>
<td>100</td>
<td>97</td>
</tr>
<tr>
<td>E. coli + Bio-Rex fraction</td>
<td>86</td>
<td>21</td>
</tr>
<tr>
<td>E. coli + Bio-Rex fraction + 2.5 mM Mg2+</td>
<td>101</td>
<td>85</td>
</tr>
<tr>
<td>E. coli + Bio-Rex fraction + 2.5 mM Ca2+</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>E. coli + Bio-Rex fraction + 5 μg heparin</td>
<td>101</td>
<td>92</td>
</tr>
</tbody>
</table>

*Values are expressed as per cent of values obtained with E. coli incubated alone.

Act D, actinomycin D.

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**Figures**

**Fig. 7.** Effect of pH on permeability-increasing and bactericidal activities of purified protein. Permeability-increasing activity was determined by incubation of 5 × 10^7 E. coli S15 (——) with 4 μg of protein (Bio-Rex fraction) in the presence (O) 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 × 10^7 E. coli with 2 μg of protein in the same incubation mixtures. The buffers used were: sodium acetate, pH 4.0 to 6.6; 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, [14C]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.

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**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 × 10^7 E. coli S15 in the standard incubation mixture in the presence (open symbols) and absence (closed symbols) of actinomycin D. Bacterical 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.
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 rabbit polymorphonuclear leukocyte fractions (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 9.5.1 Another common feature of these proteins is that they are maximally active at neutral pH (unlike the myeloperoxidase system) may fulfill their function very early 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 macro-molecular degradation and remain capable of almost unimpaired macromolecular biosynthesis for incubation periods of up to 2 h (3; 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 A, with the rabbit protein during extensive purification (1, 23). This phospholipase A, 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 granule phospholipase A, activity is low (be it at levels that are less than 5% of those in rabbit preparations), we have never observed phospholipase A, 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 granules 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 intravascular 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

### Table VI

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Permeability</th>
<th>Loss of viability</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram-negative</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>++ ++ ++ +</td>
<td>++ ++ ++ ++</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td><strong>Gram-positive</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus ferrarius</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

### 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 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 10⁶ bacteria/}

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1 Manuscript in preparation.
after intracellular sequestration of bacteria, or even concomitant 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-increasing 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 sensitive 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 bacteria show little or no binding (1). This correlation extends further to the length of the polysaccharide chain of the outer membrane lipopolysaccharides (Table VI). This and other findings (3) provide support for our concept that the primary interaction of the cationic bactericidal/permeability-increasing protein of both human and rabbit leukocytes is with surface structures, perhaps the negatively charged lipopolysaccharides, of susceptible Gram-negative bacteria. Such interaction might expose previously covered hydrophobic regions, 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 multiply (3), remains to be determined.

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REFERENCES
Purification and characterization of a potent bactericidal and membrane active protein from the granules of human polymorphonuclear leukocytes.

J Weiss, P Elsbach, I Olsson and H Odeberg