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

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JERROLD WEISS,* PETER ELSBACH,§§ INGE OLSSON,¶¶ and HÅKAN ODEBERG¶¶

From the 3Department of Medicine, New York University School of Medicine, New York, New York 10016 and the 4Department of Internal Medicine, University of Lund, Lund, Sweden

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 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 permeability of the envelope of susceptible bacteria to normally impermeant actinomycin D. 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⁺, Ca²⁺, 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 nM) produce >90% bactericidal and permeability effects on 10⁶ 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 permeability¹ (1, 3). The potency of the purified fraction towards susceptible microorganisms (10 nM produces more than 90% killing of 10⁸ 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.

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§ To whom correspondence should be addressed.

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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 1007-138 and 1807-146) were obtained from LKB-Produkter AB. Acrylamide and N,N'-methylenebisacrylamide were obtained from Eastman Kodak Co. and sodium dodecyl sulfate (se- quent purity) from Pierce Chemical Co. Sucrose (ultrapure, density grade) was acquired from Schwartz/Mann and agarse (indubiose A451 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 70,000) was obtained from Sigma. Ficol 400 was obtained from Pharmacia Fine Chemicals and Isopaque from Winthrop Laboratories. Dextrose, vitamin-free casein, nutrient broth, brain heart infusion, antibiotic Medium 3, and Freund's complete adjuvant were bought from Difco Laboratories. Hank's 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/mmol) was obtained from International Chemical and Nuclear Corporation. Both were degassed and packed under a hydrostatic pressure of 15 to 25 cm of H2O maintained by a Mariotte bottle.

Electrophoresis 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-Kromotex).

**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.

**Preparation of Granule-rich Fraction**

To obtain granules the leukocytes were resuspended in 0.34 M sucrose (2 x 10^9 cells/ml) and homogenized at 0°C 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 proteins (1, 7, 6) were compared.

**Sulfuric Acid Extraction of Whole Cells**—Polymeronucleur 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 x H2SO4 for 30 min at 0°C with periodic shaking. Neutralization of the extract by prolonged dialysis and removal of dense precipitates that form during dialysis by centrifugation at 25,000 x g for 20 min were carried out as previously described (1) except that dialysis was against 2 M 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 polymeronuclear leukocytes was performed by homogenization of the cells (1 x 10^7/ml) at 0°C 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°C or at 37°C.

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

**Chromatographic Methods**

Gel filtration chromatography was performed on Sephadex G-75 (superfine) and Sephadex G-100 (medium) at 4°C. 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 x 10^-6 cm 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.

Electrophoresis 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-Kromotex).

**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 ammonium sulfate precipitation 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 60% 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°C. The gels were destained by dialysis against several changes of a solution containing 35% ethanol and 5% glacial acetic acid.

**Amino Acid Analysis**

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

**Immunochemical 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 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-, leu-, thr-, lac-, mel+) were kindly donated by Profeesor S. Nogoa (Department of Chemistry, National Institute of Health, Tokyo, Japan); *E. coli* C600 (F+, thi-, thr-, leu-, lac+, mel+); *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.), and *E. coli* ML 308 225 by Dr. Milton Salton (Department of Microbiology, New York University School of Medicine, New York, N. Y.). *S. typhimurium* MS 395 was the gift of Dr. Mary J. Osborn (Department of Microbiology, University of Connecticut Health Center, Farmington, Conn.) and *S. typhimurium* MS 395 and rough mutants derived from it (R5 and R10) were kindly donated by Dr. Edward C. Franklin and Ms. Joan Zaretsky of New York University School of Medicine who carried out these analyses.

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2 The abbreviations used are: SDS, sodium dodecyl sulfate.

3 We are indebted to Dr. Edward C. Franklin and Ms. Joan Zaretsky of New York University School of Medicine who carried out these analyses.
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 utilis 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 triehanolamine-buffered (pH 7.75 to 7.9) minimal salts medium (11) (all E. coli strains except C8690 and both Candida strains), in nutritive broth (pH 7.5) (E. coli C8690, 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 approximately 3 to 4 h at 37°C. Stationary phase cultures of Candida, obtained from 40-h incubations, were similarly subcultured and subsequently harvested after 13 to 16 h growth at 37°C. All microorganisms were harvested and used for assay of bacterialic and permeability-increasing activities during midlate logarithmic growth phase (6 to 10 x 10⁷/ml). Microbial concentrates 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 10⁶ to 6 x 10⁷ microorganisms in a total volume of 0.4 ml of sterile physiological saline that also contained 10 μm of Tris/Cl buffer (pH 7.0), 25 μl of Hank’s solution (without phenol red), 250 μg of vitamin-free casein amino acid, and leukocyte protein fraction in the indicated amount. Incubations were carried out for 30 min at 37°C.

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 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 heat-exposed actinomycin 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 [³⁵S]leucine (0.063 μCi/μl) into cold trichloroacetic acid-insoluble material in the presence or absence of actinomycin D (12.5 μg) (1, 13). The same results are obtained using [³⁵S]leucine 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 bacterial protein 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:

\[ \text{assay for microbicidal activity} = \frac{\text{bacterial [³⁵S]leucine incorporation}}{\text{bacterial [³⁵S]leucine incorporation}} + \frac{\text{leukocyte fraction} + \text{actinomycin D}}{\text{leukocyte fraction} - \text{actinomycin D}} \times 100. \]

One arbitrary unit of permeability-increasing activity is defined as a 50% reduction by actinomycin D of [³⁵S]leucine incorporation by 2.5 x 10⁷ bacteria.

Results

The protein was determined by the method of Lowry et al. (14) using bovine serum albumin as standard. Phospholipase A₂ was assayed by using autoclaved [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).

PRELIMINARY FINDINGS: EXTRACTION OF PERMEABILITY-INCREASING ACTIVITY

An important characteristic of the highly purified bacterialic 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 μl sulfatic acid (2, 18), 0.2 M sodium acetate, pH 4.0 (8), and 0.01 μl 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 μl sulfatic 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.

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 containing <10% mononuclear cells and mononuclear cell populations containing <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 [³⁵S]leucine into bacterial protein in the presence of actinomycin D (see "Experimental Procedures").

<table>
<thead>
<tr>
<th>Table I</th>
<th>Comparison of various procedures for extraction of permeability-increasing activity from human leukocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction</td>
<td>Normal</td>
</tr>
<tr>
<td>Polymorphonuclear</td>
<td>Mononuclear</td>
</tr>
<tr>
<td>units/2.5 x 10⁷ cells</td>
<td></td>
</tr>
<tr>
<td>0.16 μl H₂SO₄/whole cell homogenate</td>
<td>22 ± 3</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
</tr>
<tr>
<td>0.2 μl acetate (pH 4.0)/granule-rich pellet</td>
<td>8.8 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
</tr>
<tr>
<td>0.01 μl citric acid/whole cell homogenate</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
</tr>
</tbody>
</table>

After 30-min incubation, 10-μl samples were taken from the incubation mixtures, added in trichloroacetic acid-Pasteur plates on either nutrient agar (E. coli, S. typhimurium, Bacillus 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°C overnight (all bacterial strains except M. lysodeikticus), 48 h (Candida), or 2 to 3 days at room temperature (M. lysodeikticus).
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 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 the 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 × 10⁹ *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 × 10⁹ *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 pl 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...
Fig. 3. Isoelectric focusing of bactericidal/permeability-increasing (PI) fraction obtained by Bio-Rex 70 chromatography. The active fraction (2.8 mg of protein) was dialyzed against a mixture of Ampholines (0.5%) with pH ranges of 7 to 9 and 9 to 11 and applied in the middle of a 110-ml column containing a continuous sucrose gradient (0 to 46%) and a constant 1% Ampholine concentration of the above Ampholine mixture. Focusing was begun at 300 V (0.5 watts) and continued for 48 h at 700 V. The column was maintained at 4°C by circulating cooled running water. After termination of focusing, fractions of 3 ml (1 to 20; 45 to 60) or 1 ml (21 to 44) were collected by slowly emptying the column through the bottom outlet and assayed for pH. Bactericidal and permeability-increasing activities toward E. coli S15 and protein of the fractions were determined as described under "Experimental Procedures" after exhaustive dialysis against distilled water to remove Ampholines. Protein recovery was approximately 80%.

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.

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).

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

Leukocytes, 2.5 x 10⁶, obtained from a chronic myelocytic leukemia patient were used as starting material for purification. Protein, permeability-increasing, and bactericidal activities were determined as described under "Experimental Procedures." The values shown represent the mean of at least three independent assays carried out in duplicate.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein (mg)</th>
<th>Permeability-increasing activity (units/mg)</th>
<th>Bactericidal activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 4.6 extract of granules</td>
<td>264 (100)</td>
<td>600 (100)</td>
<td>2.0 (50)</td>
</tr>
<tr>
<td>Sephadex G-75 chromatography</td>
<td>14.4 (5.4)</td>
<td>645 (107)</td>
<td>45 (40)</td>
</tr>
<tr>
<td>Bio-Rex chromatography</td>
<td>4.0 (1.5)</td>
<td>356 (59)</td>
<td>85 (38.1)</td>
</tr>
</tbody>
</table>

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

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

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.
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 tentatively 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 lipopolysaccharides 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.

![FIG. 7. Effect of pH on permeability-increasing and bactericidal activities of purified protein. Permeability-increasing activity was determined by incubation of 5 × 10⁷ E. coli S15 with 4 µg 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 × 10⁷ E. coli with 2 µg 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, [¹⁴C]leucine incorporation by E. coli incubated alone is markedly depressed. Results are expressed as percent of E. coli incubated alone at the indicated pH and represent the mean of two closely similar experiments carried out in duplicate.](image)

![FIG. 8. Effect of heat treatment on (A) permeability-increasing and (B) bactericidal activities of purified proteins. 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 × 10⁷ 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 percent of E. coli incubated alone and represent the mean of two closely similar experiments.](image)
Bactericidal Protein from Human Leukocytes

TABLE VI
Susceptibility of different microorganisms to permeability-increasing and microbicidal effects of purified human polymorphonuclear leukocyte fractions

<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>S15 (r)</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>S17 (r)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B600 (r)</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>H0129 (s)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ML 308 225 (s)</td>
<td>N.T.</td>
<td>+</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-30 (Rc)</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>G-30 + galactose (s)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R10-395 (Rd)</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>R5-395 (Rb)</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>MS-395 (s)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Gram-positive</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
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<td></td>
</tr>
<tr>
<td>Qui</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>W46</td>
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<td>0</td>
</tr>
<tr>
<td>C1</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>Ferrari (coagulase +)</td>
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</tr>
<tr>
<td>Streptococcus faecalis</td>
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</tr>
<tr>
<td>B55</td>
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</tr>
<tr>
<td>B. subtilis</td>
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<td>0</td>
</tr>
<tr>
<td>M. lysodeikticus</td>
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</tr>
<tr>
<td>Fungi</td>
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<td></td>
</tr>
<tr>
<td>Candida albicans</td>
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</tr>
<tr>
<td>Candida parapsilosis</td>
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</table>

**DISCUSSION**

The bactericidal protein isolated in nearly pure form from human polymorphonuclear leukocyte 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, 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 9.5.1 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 10^7 bacteria/mL) 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 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 granule also contain phospholipase A2 activity (be it at levels that are less than % 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 cytoplasma is not known. In a recent in vitro study the bactericidal activity of the myeloperoxidase/H2O2 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 extract 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
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 Canda 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 (2) 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


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