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Cationic Antimicrobial Proteins Isolated from Human Neutrophil Granulocytes in the Presence of Diisopropyl Fluorophosphate

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Acid (0.2 M sodium acetate, pH 4.0) extracts of granules recovered from disrupted human polymorphonucleur granulocytes (PMNs) exhibited in vitro antimicrobial activity against *Salmonella typhimurium*. To minimize proteolytic destruction or modification of antimicrobial proteins derived from these granules, we pretreated the PMNs with the serine protease inhibitor diisopropyl fluorophosphate. Fractionation of such extracts by carboxymethyl Sephadex and Sephadex G-75 chromatography resulted in the recovery of at least two antimicrobial, cationic proteins. These proteins differed substantially in antimicrobial activity, amino acid composition, and molecular weight (M_r, 37,000 and 57,000). As we have shown before (Shafer et al., Infect. Immun. 43:834–858), with unfractionated proteins, these two proteins exhibited diminished activity against a polymyxin B-resistant (PB^r) mutant of *S. typhimurium* compared with their activity against the isogenic parental polymyxin B-sensitive (PB^s) strain. Expression of the relevant mutation (*prmA*) in the PB^r mutant decreases the electronegativity of lipid A, owing to increased 4-amino-4-deoxy-L-arabinosylation at the 4′ phosphate residue (Vaara et al., FEBS Lett. 129:145–149). The data suggest that at least two different cationic proteins account for the antimicrobial capacity of extracts from human PMN granules. Moreover, the availability of anionic charges in the outer membrane of *S. typhimurium* due to free lipid A phosphates apparently dictates phenotypic levels of resistance to both of the cationic proteins extracted from human PMN granules.

Cationic proteins (8, 10, 12, 13, 15, 21–30) derived from polymorphonuclear granulocyte (PMN) cytoplasmic granules appear likely to mediate oxygen-independent, intraleukocytic killing of bacteria (7). Earlier studies (8, 12, 13, 15, 22) dealing with human PMNs have documented the role of lipopolysaccharide (LPS) in the determination of the phenotypic level of resistance of gram-negative bacteria to both granule extracts and more-purified cationic antimicrobial proteins (CAPs); that is, deep rough mutants containing truncated LPS were less resistant to CAPs than was the isogenic smooth parental strain.

We (15) recently proposed that deep rough LPS mutants of *Salmonella typhimurium* exhibit decreased resistance to CAPs from human PMN granules as a result of increased exposure of a receptor localized within the hydrophobic, anionic lipid A component of LPS. This suggestion was supported by the observation that a polymyxin B-resistant (PB^r) mutant of *S. typhimurium* LT-2 which exhibited a fourfold increase in substitution of 4-amino-4-deoxy-L-arabinose at the 4′ lipid A phosphate (19) was phenotypically more resistant to CAP than was the parental strain. Accordingly, we proposed that the decreased electronegativity provided by the relevant mutation (*prmA*) increased levels of resistance to CAP.

To further test this hypothesis and to elucidate the number of proteins accounting for this behavior, we sought to obtain PMN antimicrobial proteins in a more purified form. In this communication, we report the recovery of two different CAPs from human PMN granules under conditions of serine protease inhibition. The proteins exhibited different degrees of antimicrobial activity in vitro against *S. typhimurium*. Most important, we found that the *prmA* mutation increased the resistance of *S. typhimurium* to these CAPs.

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MATERIALS AND METHODS

Source of PMNs and preparation of granule extracts. A granulocyte concentrate (95% PMNs) was obtained with leukophoresis and informed consent from a person with chronic myleocytic leukemia undergoing treatment at Emory University Hospital, Atlanta, Ga. PMNs were prepared and disrupted by homogenization as described by Rest et al. (12). Mixed (specific and azurophilic) granules were harvested by differential centrifugation as described previously (12, 13) and extracted at 4°C with 0.2 M sodium acetate (pH 4.0). Granule debris was collected by high-speed centrifugation at 20,000 × *g* for 30 min. The protein concentration of crude granule extracts was determined by the method of Bradford (2) with chicken egg white lysozyme as the standard. The clarified supernatant was stored at −80°C.

Analysis of serine protease activity. PMNs were pretreated with diisopropyl fluorophosphate (DFP) as described by Amrein and Stossel (1). Enzymatic assays for elastase and cathepsin G were performed with crude granule extracts as described by Starkey and Barrett (17) and Vaara et al. (20). The substrate employed for detection of cathepsin G activity was *N*-benzoyl-DL-phenylalanine-2-napthyl ester; *N*-benzylocarbonyl-L-alanine-2-naphthyl ester was employed for measurements of elastase activity.

Fractionation of PMN granule extracts. Granule extracts were dialyzed overnight at 4°C against 0.05 M sodium acetate (pH 5.0)–0.15 M sodium chloride. The dialyzed crude granule extract was applied to a carboxymethyl Sephadex (C-50; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) column (1.25 by 25 cm) which had been previously equilibrated with 0.05 M sodium acetate (pH 5.0)–0.15 M sodium chloride. The column was extensively washed (200 ml) with this buffer. The bound protein was eluted with a two-step linear salt gradient. The salt gradients (400 ml each)

consisted of 0.15 to 0.4 M and 0.4 to 1.0 M sodium chloride in 0.05 M sodium acetate (pH 5.0). The column was then extensively washed with 6 M urea in 0.05 M sodium acetate (pH 5.0)–1.5 M sodium chloride. Protein elution was monitored by measuring absorbance at 280 nm with a Cary 14 spectrophotometer, and salt concentrations were determined by conductivity measurements. Appropriate fractions were pooled and stored at 4°C before being tested for antimicrobial activity (see below).

The antimicrobial preparation obtained by ion exchange chromatography was dialyzed overnight at 4°C against 0.2 M sodium acetate (pH 4.0) and concentrated by membrane ultrafiltration at 4°C with a YM-5 membrane filter (Amicon Corp., Lexington, Ky.). Approximately 3.3 ml of protein (total volume, 0.3 ml) was applied to a Sephadex G-75 (SF; Pharmacia) column (0.5 by 50 cm) which had been equilibrated with 0.2 M sodium acetate (pH 4.0). Fractions of 0.25 ml were collected and diluted to 1.0 ml with 0.2 M sodium acetate (pH 4.0), and the absorbance at 280 nm of each fraction was determined.

Bacterial strains and antimicrobial assays. *S. typhimurium* SL-1004 (Rd_1 LPS chemotype) was employed as the primary test strain in microbicidal assays. *S. typhimurium* SH9178 (Rb LPS chemotype) and the isogenic PB^r derivative SH7426 (also Rb LPS chemotype) were also employed. These latter strains differ in levels of PB^r (18–20). As a result of a mutation at the *prmA*$^+$ locus, the PB^r strain (*prmA*) has a fourfold increase in the amount of 4-amino-4-deoxy-L-arabinose at the partially substituted 4′ lipid A phosphate (19).

Antimicrobial assays were routinely performed essentially as described by Rest et al. (12). Bacteria grown overnight in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) were diluted 100-fold in fresh broth and grown at 37°C with shaking until a cell density of 5×10^8 CFU/ml was reached. These cultures were pelleted by centrifugation at $2,000 \times g$, washed once with tryptone saline (12), and diluted to 1×10^3 to 5×10^3 CFU/ml. Microbicidal assays were preformed in sterile microtiter trays. All granule protein fractions to be tested were dialyzed at 4°C against sterile distilled water before being tested to remove the salt present in the buffer. After the addition of appropriate amounts of granule protein (final volume adjusted to 0.1 ml with tryptone saline), bacterial suspensions (0.1 ml) were added and incubation was allowed to proceed for 1 h at 37°C. Controls consisted of incubation of bacteria in the absence of granule protein. After incubation, 0.1- and 0.01-ml aliquots were plated onto Trypticase soy agar plates. The percentage of bacteria killed was determined by the following equation: % killed = 100 × (bacteria incubated with protein)/(bacteria incubated alone).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions with a 15% separating gel which contained a 4% stacking gel as described by Laemmli (6). The ratio of acrylamide to bisacrylamide was 37.5. Electrophoresis was carried out at 75 V until the bromophenol blue dye entered the separating gel, at which time the voltage was increased to 150 V. Electrophoresis was allowed to proceed until the tracking dye reached the bottom of the separating gel. The gel was removed and washed overnight in 40% methanol–10% acetic acid, washed twice (30 min each) in 10% ethanol–5% acetic acid, and then oxidized and silver stained by the protocol described by the supplier (BioRad Laboratories, Richmond, Calif.).

Amino acid analysis. A 100-μg amount of protein in glass distilled water was hydrolyzed with HCl (final concentration, 6 N) at 100°C for 24 h. Analyses were performed in a JEOL 6AH amino acid analyzer.

RESULTS

DPF inhibition of PMN granule serine protease activity. Human granule proteins exhibiting antimicrobial activity in vitro against *S. typhimurium* have been isolated by Weiss et al. (22) and Modzrakowski and Spitznagel (8). The protein isolated by Weiss et al. exhibited an M_r of 59,000, whereas that isolated by Modzrakowski and Spitznagel had a an M_r of 36,500. Because neither group reported the isolation of both proteins, we sought to determine whether the apparent discrepancy in the reported molecular weights was due to modification of CAP by endogenous PMN serine proteases. Earlier studies by Amrein and Stossel (1) suggested that the destruction and modification of several PMN proteins owing to the action of endogenous serine proteases could be prevented if the PMNs were pretreated with the potent serine protease inhibitor DFP. Accordingly, granule extracts obtained from DFP-treated and control granulocytes were compared for differences in serine protease activity, protein

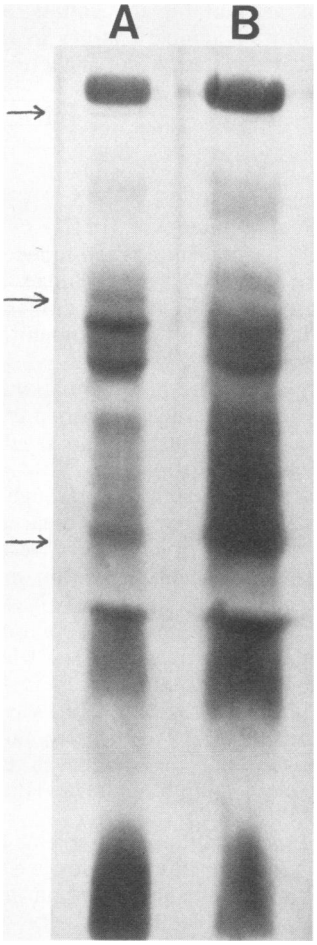

FIG. 1. Comparison of the protein composition of PMN granule extracts obtained from DFP-treated (A) and control (B) PMNs. A total of 25 μg of protein of each extract was solubilized under reducing conditions and electrophoresed in 15% polyacrylamide gels. Protein bands were visualized by silver staining. The arrows amplify the minor differences between these preparations. The band indicated by the top arrow did not photograph clearly.

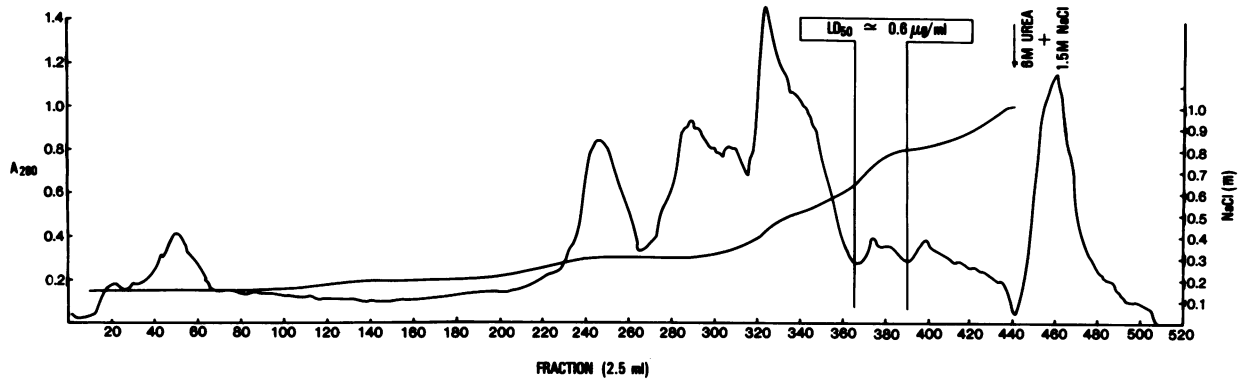

FIG. 2. Partial purification of CAPs from crude granule extracts by carboxymethyl Sephadex chromatography. A total of 350 ml of protein was applied to the column, and protein was eluted as described in the text. The column flow rate was ca. 10 ml/h. The line extending from fractions 1 through 510 indicates the absorbance at 280 nm (A_{280}) of each fraction; the line extending from fractions 10 through 440 indicates the salt concentration of each fraction. The recovery of protein was 99.4%. The predominate antimicrobial fraction was localized between fractions 365 through 390.

content, and antimicrobial activity. As reported by Amrein and Stossel (1), DFP readily inhibited the enzymatic activity of the major granule-derived serine proteases (elastase and cathepsin G) as judged by the ability of control but not DFP-treated extracts to hydrolyze the relevant synthetic substrates (data not shown). Some minor differences were noted in the protein composition of control and DFP-treated extracts when they were analyzed by SDS-PAGE (Fig. 1). Nevertheless, both extracts killed *S. typhimurium* SL-1004 equally; after overnight dialysis at 4°C, the approximate 50% lethal dose (LD_{50}) for both extracts was 10 μg of protein per ml.

Fractionation of antimicrobial activity from crude granule extracts. We applied 350 ml of crude granule antimicrobial protein, dissolved in 0.05 M sodium acetate (pH 5.0)–0.15 M sodium chloride, to the carboxymethyl Sephadex column. The absorbed protein, eluted with a two-step linear salt gradient (see Materials and Methods), emerged from the column in several peaks (Fig. 2). Titration of the antimicrobial activity in pooled fractions demonstrated that the antimicrobial activity was located in fractions 365 through 390. As little as 0.6 μg of protein per ml killed 50% of the exposed bacteria. Pooled fractions (340 through 364 and 391 through 440) adjacent to the predominant antimicrobial pool killed *S. typhimurium* SL-1004 to a much lesser extent (LD_{50}, 20 μg of protein per ml), whereas other pooled fractions (pooled fractions eluted with high salt, urea, and fractions eluted with salt concentrations of <0.6 M NaCl) exerted little antimicrobial activity (LD_{50}, >20 μg of protein per ml).

The antimicrobial fraction had ca. 1.6% (5.6 ml) of the protein originally applied to the carboxymethyl Sephadex column. The protein composition of this material was resolved into several bands (M_r, 19,000 to 60,000) when SDS-PAGE profiles were analyzed by silver staining (see Fig. 5, lane C).

The potent antimicrobial protein preparation obtained with carboxymethyl Sephadex chromatography yielded several fractions with Sephadex G-75 chromatography. The profile shown in Fig. 3 resulted when 3.3 mg of the partially purified antimicrobial protein was applied to such a column. To reduce contamination, we analyzed individual peak fractions for antimicrobial activity. Antimicrobial activity was localized in peak fractions B and C. The approximate LD_{50} for peak fraction B was 0.25 μg of protein per ml, and that of peak fraction C was 6.0 μg of protein per ml (Fig. 4).

The protein composition of peak fractions B and C was determined by silver staining of SDS-PAGE profiles. Before electrophoresis, protein samples were dialyzed against glass distilled water and then solubilized under reducing conditions. The results (Fig. 5) indicate that peak fraction B (lane B) contained an intensely stained band of with a molecular weight of ca. 57,000. Two additional and much less intense bands (molecular weight, 58,000 and 56,000) were also apparent, but only when the gels were overloaded with 250 ng of protein. Peak fraction C was found to contain a diffuse band with a molecular weight of ca. 37,000 (Fig. 5, lane A). A summary of the isolation and antimicrobial activities of these proteins is shown in Table 1.

The amino acid composition of the antimicrobial proteins was determined (Table 2). Amino acid analysis was carried out with 100 μg of protein obtained with pooled fractions as described in the legend to Fig. 3. Although both protein samples contained high concentrations of nonpolar (ca. 50%) and cationic (ca. 15%) residues, they differed significantly in the content of proline, glycine, methionine, isoleucine, tyrosine, lysine, and arginine.

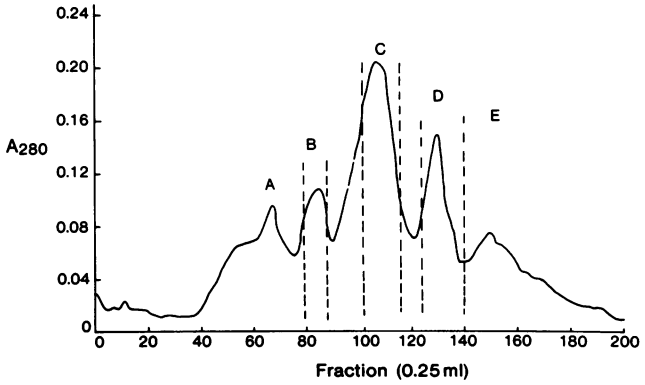

FIG. 3. Isolation of CAPs by Sephadex G-75 chromatography from the partially purified CAP preparation. Approximately 3.3 ml of protein in 0.2 M sodium acetate (pH 4.0) was applied to the column. The column flow rate was ca. 0.66 ml/h. The predominate peaks (A through E) were tested for antimicrobial activity and protein composition. These fractions, designated by the broken lines, were subsequently pooled for amino acid analysis. A_{280}, Absorbance at 280 nm.

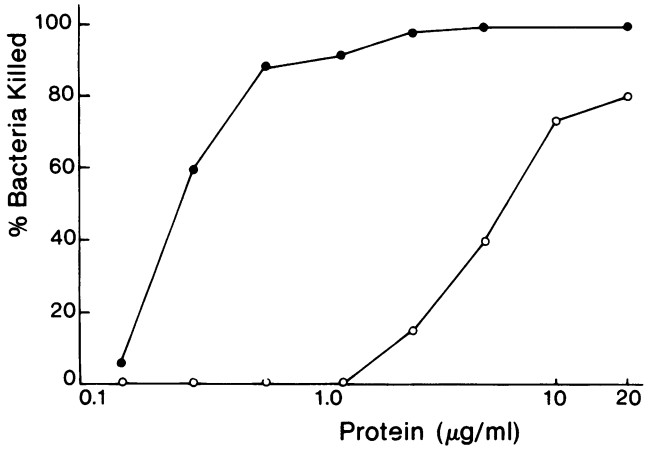

FIG. 4. Antimicrobial activity of the 37,000- and 57,000-dalton CAPs obtained by Sephadex G-75 chromatography. Activities of the 37,000-dalton protein (○) (LD_{50}, ca. 6 μg/ml) and the 57,000-dalton protein (●) (LD_{50}, ca. 0.25 μg/ml) are shown.

Increased resistance of a rough mutant of *S. typhimurium* to CAP owing to *prmA*. We wished to determine whether the *prmA* mutation altered the resistance of *S. typhimurium* to both CAPs in a manner observed previously (15) with a partially purified CAP preparation. Accordingly, we examined the phenotypic levels of resistance of isogenic strains, which are reported (18, 19, 23) to differ only at the *prmA*$^+$ locus. In three separate microbicidal assays (Fig. 6), we observed that SH7426 (*prmA*) was at least three- to fourfold more resistant to both CAPs than was the parental strain, SH9178 (*prmA*$^+$).

DISCUSSION

That PMNs obtained from patients with chronic granulomatous disease and maintained aerobically (14, 27) and PMNs from normal persons (7, 11) or rabbits (27) maintained under strict anaerobic conditions readily ingested and killed certain bacteria suggested that PMN microbicidal systems can function in the absence of oxidative processes. Accordingly, it was relevant to examine both the PMN and bacterial factors which influence the efficacy of oxygen-independent microbicidal systems.

The involvement of cationic proteins derived from the cytoplasmic granules in killing bacteria has been recognized in the PMN systems of rabbits (3, 21, 23–29), guinea pigs (30), rats (5), cows (4) and humans (8, 10, 22, 25, 27, 28). However, the biochemical nature of such proteins derived from all PMN species have remained uncertain. Weiss et al. (22) reported the purification from crude, human PMN granule extracts of a cationic protein with in vitro antimicrobial activity against *S. typhimurium* and *Escherichia coli*. This protein migrated as a single entity in SDS-PAGE with an M_r of 59,000. In relatively low concentrations it was antimicrobial. In substantially larger concentrations it appeared to increase outer membrane permeability. It was therefore termed bactericidal permeability-increasing (BPI) protein. Subsequently, Modrzakowski and Spitznagel (8) reported the isolation from crude granule extracts of a 36,500-dalton protein that also exhibited antimicrobial activity in vitro against *S. typhimurium* and *E. coli*. Both of these proteins were more active against rough and semi-rough LPS mutants than they were against fully smooth bacteria. In an earlier communication (15), we proposed that such mutants were phenotypically less resistant to a partially purified antimicrobial preparation than was the smooth parent, owing to loss of the hydrophilic O and core polysaccharides with consequent exposure of anionic groups of lipid A. Two lines of evidence support this hypothesis. Target organisms preincubated with sublethal concentrations of polymyxin B, a cationic antibiotic known to interact with lipid A (9), rendered the bacteria phenotypically resistant to granule proteins. A mutation (*prmA*) which afforded increased resistance to polymyxin B (18–20) and increased (by three- to fourfold) 4-amino-4-deoxy-L-arabinosylation at the partially substituted 4′ lipid A phosphate (19) resulted in increased resistance to partially purified granule protein (15).

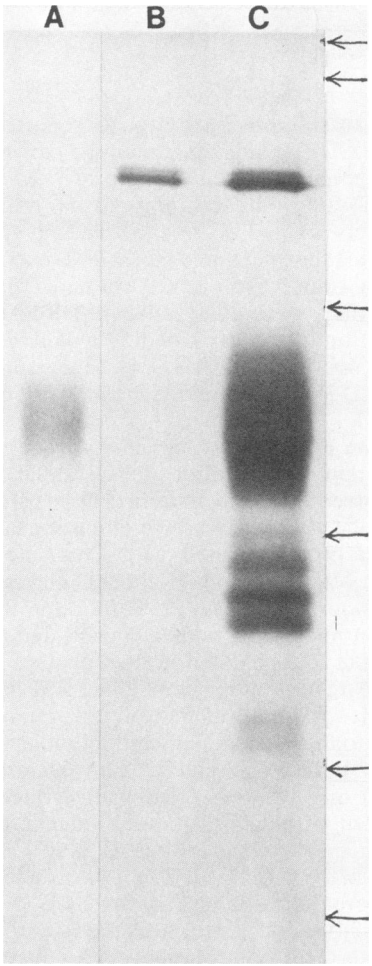

FIG. 5. Electrophoretic mobility of CAPs in SDS-PAGE after carboxymethyl Sephadex and Sephadex G-75 chromatography. Proteins were solubilized under reducing conditions and analyzed for differences in electrophoretic mobility as described in the legend to Fig. 1. (A) A total of 0.5 μg of the 37,000-dalton protein obtained after Sephadex G-75 chromatography; (B) 0.5 μg of the 57,000-dalton protein obtained after Sephadex G-75 chromatography; (C) 10 μg of the partially purified CAP preparation obtained after carboxymethyl Sephadex chromatography. Molecular weight estimates were determined by analysis of the mobility of molecular weight standards: phosphorylase B, 92,500; bovine serum albumin, 66,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100; and lysozyme, 14,000. The arrows indicate the electrophoretic mobility of these six standards.

To study the molecular basis for the effect of lipid A on the resistance of *S. typhimurium* granule proteins, we sought antimicrobial proteins in a more purified form. We initially attempted to use the purification scheme described by Weiss et al. (22) for the preparation of antimicrobial proteins from PMN granules. In several different experiments, we were unable to obtain CAP by the procedure of these authors. Partially purified antimicrobial preparations obtained with Sephadex G-75 chromatography often precipitated when dialyzed overnight against the buffer recommended for subsequent chromatography on BioRex 70. When significant precipitation did not occur and protein was applied to the BioRex column and eluted as described previously (22), we were unable to account for greater than 50% of the applied protein. Moreover, antimicrobial activity could be recovered only when the column was extensively washed with 1.5 M sodium acetate in 6 M urea. Even under these conditions, we were unable to account for at least 30% of the applied protein.

We describe here the successful procedure we have devised for the isolation of CAP. The 0.05 M sodium acetate (pH 5.0)–0.15 M sodium chloride buffer we used for dialysis of crude extracts and ion exchange chromatography did not cause precipitation. Subsequent chromatography of crude extracts over carboxymethyl Sephadex resulted in the recovery of virtually all of the applied protein. Moreover, elution of antimicrobial activity did not require the use of 6 M urea or other denaturing reagents. The antimicrobial preparation obtained with carboxymethyl Sephadex contained ca. 1.6% of the protein applied to the column and was at least 16-fold more active than the crude extract. Chromatography of this preparation over Sephadex G-75 resulted in the recovery of not just one but two CAPs. The 57,000-dalton CAP which we obtained after molecular sieve chromatography of the partially purified CAP preparation exhibited the antimicrobial properties and amino acid composition resembling those reported by Weiss et al. for human BPI protein (22). A 37,000-dalton CAP that differed substantially from the 57,000-dalton CAP in amino acid composition and antimicrobial activity was also recovered from crude granule extracts. The molecular weight of this CAP was closely similar to that of the CAP isolated by Modrzakowski and Spitznagel (8). More recently, from non-DFP-treated leukemic and normal PMNs (data not shown), we have obtained electrophoretically identical CAPs which exhibit in vitro antimicrobial activity against *S. typhimurium*.

TABLE 1. Summary of isolation of CAPs from human PMN granule extracts

Source of protein	% Recovery of total protein	Antimicrobial protein (ml)[a]	LD_{50} (μg of protein per ml)[b]
Crude extract		350	10
After carboxymethyl Sephadex	99.4	5.6 (1.6)	0.6 (16.6)
After Sephadex G-75	93.4		
Pool B		0.145 (0.04)	0.25 (40)
Pool C		1.612 (0.46)	6 (1.6)

[a] Numbers in parentheses represent the percentage of crude extract. The values for pools B and C are probably underestimated in that 58.9% of the antimicrobial preparation obtained from the carboxymethyl Sephadex column was applied to the Sephadex G-75 column.

[b] Numbers in parentheses represent the fold change in antimicrobial activity as compared with crude extract.

TABLE 2. Amino acid composition of human PMN granule antimicrobial proteins

Amino acid	% of total amino acids in:	
	57,000-dalton protein	37,000-dalton protein
Aspartic acid	9.3	10.5
Threonine	4.5	5.4
Serine	8.5	7.3
Glutamic acid	9.4	9
Proline	6.1	12.4
Glycine	6.3	10.7
Alanine	6.4	6.3
Valine	6.9	6.7
Methionine	2.6	1.1
Isoleucine	5.5	2.2
Leucine	10.4	7.2
Tyrosine	2.7	0.6
Phenylalanine	5.7	4.2
Histidine	4.4	5.1
Lysine	7.8	1.6
Arginine	3	8.9
Half-cystine	ND[a]	ND

[a] ND, Not detected.

The degree of purity of the CAPs isolated in this investigation was assessed by silver staining of SDS-PAGE profiles. In silver stained SDS-PAGE profiles (not shown), we found that we could detect as little as 50 ng of the 57,000-dalton protein. The gel profile presented in Fig. 5, lane B resulted from an application of 500 ng of the protein and, hence, represents an overload. The contaminants which are readily apparent were detected only when 10 times the amount of detectable 57,000-dalton protein was solubilized. We are currently attempting to achieve a greater degree of purity of this CAP by high-pressure liquid chromatography. The purity of the 37,000-dalton CAP was also assessed by silver staining of SDS-PAGE profiles. We were unable to detect a silver-stainable band in the 57,000-dalton range, even when 5 μg of protein was solubilized. Accordingly, we believe that the 37,000-dalton CAP is contaminated by the 57,000-dalton CAP by less than 1%. On the basis of this estimate, we calculate that the amount (6 μg/ml) of the 37,000-dalton CAP required to achieve an LD_{50} would contain less than 0.06 μg of the 57,000-dalton CAP per ml. This value is four times less than that required to achieve an LD_{50} for the 57,000-dalton CAP (Fig. 4).

On a weight basis, both CAPs represent relatively minor constituents of the protein in crude granule extracts (Table 1). Weiss et al. (22) obtained a similar result for their 59,000-dalton CAP (BPI protein). They indicated that BPI represented ca. 1.5% of the total protein present in crude granule extracts. Although the 57,000-dalton CAP isolated in this study resembles BPI in mobility in SDS-PAGE profile, amino acid composition, and antimicrobial activity, we estimate, on the basis of total recovery (Table 1), that this protein represents less than 0.1% of the total protein content. Since we were unable to successfully employ the purification protocol described by Weiss et al., it is difficult to explain the difference in yield of the 57,000-dalton CAP. If the 57,000-dalton CAP and BPI protein are identical, the differences in overall yields might be due to differences in PMN source or loss during concentration, dialysis, and chromatography. The 37,000-dalton CAP was found (Table 1) to constitute ca. 0.4% of the total crude granule extract. Although against *S. typhimurium* it was ca. 24-fold less active on a weight basis than the 57,000-dalton CAP, the

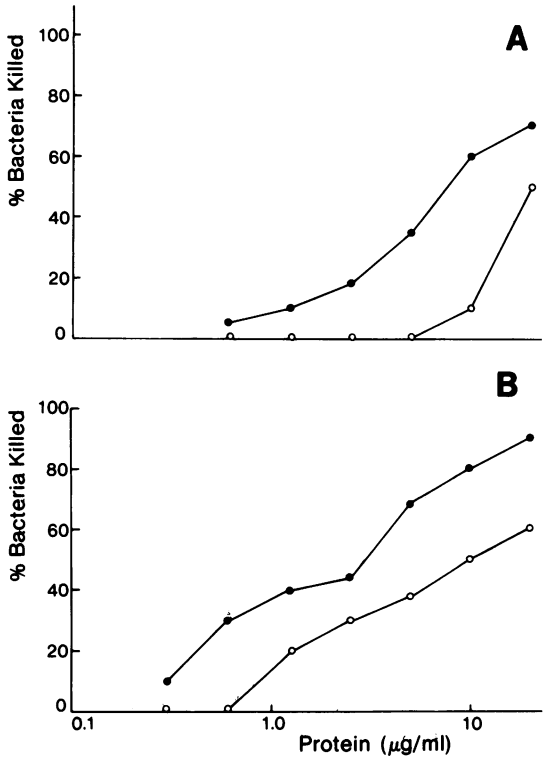

FIG. 6. A mutation (*prmA*) affecting resistance to polymyxin B afforded increased resistance to CAPs derived from human PMN granule extracts. (A) Sensitivity of strain SH9178 (*prmA*$^+$; LD_{50}, ca. 7.5 μg/ml) (●) and SH7426 (*prmA*; LD_{50}, ca. 20 μg/ml) (○) to the 37,000-dalton protein. (B) Sensitivity of SH9178 (*prmA*$^+$; LD_{50}, ca. 3 μg/ml) (●) and SH7426 (*prmA*; LD_{50}, ca. 10 μg/ml) (○) to the 57,000-dalton protein. Both strains synthesized an LPS of the Rb chemotype. Each data point is the average of triplicate samples; values did not differ by more than 5%.

difference in yield (0.4 versus 0.04%) suggests that this protein might contribute significantly to the O_2-independent antimicrobial arsenal of PMN.

Our earlier studies (15) suggested that the known interaction of polymyxin B and lipid A (9) could serve as a model for studying lipid A interactions with CAPs derived from PMN granules. The data presented in Fig. 6 indicate that the *prmA* mutation results in increased resistance to the isolated granule cationic antimicrobial granule proteins, much as it does to polymyxin B (19). Thus, the decrease in lipid A electronegativity provided by *prmA* presumbably diminishes the capacity of lipid A for binding cationic granule proteins in a manner perhaps similar to that described (9, 20) for lipid A and polymyxin B. Since the *prmA* mutation does not affect the diffusion of either hydrophobic or hydrophilic compounds through the outer membrane (18), it is possible that increased resistance of the *prmA* strain to granule proteins is due largely to decreased binding of cationic proteins, implying that the CAPs are bound to the microbial surface by ionic bonds.

The data obtained in this investigation indicate that at least two granule proteins of different antimicrobial activities contribute to the antimicrobial activity of granule extracts in vitro. Whether these proteins similarly exert antimicrobial activity in the environment provided by the phagolysosome remains to be determined.

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