Azurocidin and a Homologous Serine Protease from Neutrophils

Differential Antimicrobial and Proteolytic Properties

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Abstract

Two 29-kD polypeptides, azurocidin and p29b, were purified to homogeneity from human neutrophils by acid extraction of azurophil granule membrane–associated material followed by gel filtration and reverse-phase chromatography. Azurocidin and p29b share NH_2-terminal sequence homology with each other as well as with elastase, cathepsin G, and other serine proteases. p29b bound [^{3}H]diisopropyl fluorophosphate and hydrolyzed elastin, casein, and hemoglobin. A peptide substrate for p29b could not be identified. Azurocidin neither bound [^{3}H]diisopropyl fluorophosphate nor hydrolyzed any of the proteins, peptides, or esters tested. In microbicidal assays, purified azurocidin was comparable to p29b in activity against *Escherichia coli, Streptococcus faecalis,* and *Candida albicans.* The antimicrobial activity of azurocidin was enhanced under mildly acidic conditions, but was inhibited in a dose-dependent manner by NaCl, $CaCl_2$, or serum. Immunoblot analysis with monospecific antibodies localized > 90% of the azurocidin and > 75% of the p29b to azurophil granule-rich fractions of PMN lysates. Immunoelectron microscopy confirmed the localization of azurocidin to the azurophil granules. Azurocidin associated with the azurophil granule membrane, but did not appear to be an integral membrane protein. Thus, azurocidin and p29b are members of a family of serine protease homologs stored in azurophil granules and may play a role in inflammatory and antimicrobial processes involving PMN. (*J. Clin. Invest.* 1990. 85:904–915.) polymorphonuclear leukocyte • lysosomes • granules • antibiotic • esterase

Introduction

Azurophil granules, specialized lysosomes of the neutrophil, contain neutral serine proteases implicated in the killing and digestion of microbes and the destruction of extracellular matrix proteins (1, 2). Cathepsin G and elastase, two of these serine proteases, possess antimicrobial activity independent of their proteolytic activity (3, 4). A recently purified neutrophil serine protease named proteinase 3 is active against elastin; its potential antimicrobial activity was not studied (5).

We recently reported the fractionation of the protein components of the azurophil granule membrane associated material by reverse-phase chromatography, their identification by NH_2-terminal sequence analysis, and a quantitative comparison of their microbicidal activities (6). In that study, two apparently novel 29-kD polypeptides were identified whose NH_2-terminal sequences were highly homologous to elastase and cathepsin G. Antimicrobial activity was associated with both of the 29-kD proteins, named azurocidin and p29b. The azurocidin-rich fraction was the more potent, but it also contained a minor protein of 80 kD. This report describes the purification to homogeneity of azurocidin, the antimicrobial and proteolytic activities of azurocidin and p29b, and their subcellular localization to the azurophil granules of human PMN.

Methods

Isolation and subcellular fractionation of PMN. Blood from healthy donors was processed as previously described (7). 450 ml of blood yielded 10^9 cells that were > 99% PMN. Typically, 93–97% of the PMN were neutrophils and 3–7% were eosinophils. The PMNs were lysed by nitrogen cavitation, and the azurophil granules were separated on discontinuous Percoll density gradients as described (7), except that treatment with diisopropyl fluorophosphate (DFP)[1] was omitted in preparations to be assayed for proteolytic activity. Granules were stored in relaxation buffer (7) at −70°C.

Extraction of azurophil granules. Azurophil granule membrane associated material (referred to as granule membranes) was obtained by seven freeze-thaw cycles in dry ice/acetone with a 15-s sonication pulse between each cycle. After centrifugation at 10,000 *g* for 60 min at 4°C, the pelleted material was extracted with 50 mM glycine, pH 2.0 for 40 min at room temperature. The extract was centrifuged at 10,000 *g* for 20 min at 4°C, and the acid soluble supernatant was concentrated 20-fold with a Centricon-10 microconcentrator of 10,000 M_r cutoff (Amicon Corp., Danvers, MA). The acid extract of the azurophil granule membrane was stored in aliquots at −70°C. Protein concentrations were determined by the method of Lowry et al. (8) using BSA as a standard.

Purification of azurocidin and p29b. 1 mg of the acid extract was applied to a Bio-Sil TSK-125 size exclusion column (Bio-Rad Laboratories, Richmond, CA) equilibrated in 50 mM glycine, pH 2.2, 100 mM NaCl, and eluted at 0.5 ml/min on an HPLC system (Waters/Millipore, Milford, MA) (9). Absorbance at 280 nm was monitored and antimicrobial activity was assayed as described below. Peaks to be further purified by reverse-phase HPLC were brought to 0.1% TFA and

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1. *Abbreviations used in this paper:* BPI, bactericidal/permeability-increasing protein; CAP, cationic antimicrobial protein; DFP, diisopropyl fluorophosphate; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; TFA, trifluoroacetic acid.

applied to a Vydac wide pore (250 × 4 mm) C4 column (Rainin Instruments, Emeryville, CA) equilibrated in 0.1% TFA. Proteins were eluted at 1 ml/min with a linear gradient of 0–100% acetonitrile in 0.1% TFA and monitored for absorbance at 214 nm. Fractions were dried in a Speed-vac concentrator (Savant Instruments, Farmingdale, NY), rinsed with 0.1% acetic acid, dried, and resuspended in 10 mM $NaPO_4$, pH 5.5. For samples to be tested for antimicrobial activity, BSA (fraction V, 98–99% albumin; Sigma Chemical Co., St. Louis, MO) was added to the reverse-phase fraction at a final concentration of 0.02% before drying (6).

SDS-polyacrylamide gel electrophoresis. SDS-PAGE was performed as described by Laemmli (10) on 10% or 15% slab gels. Before electrophoresis, samples were boiled in sample buffer (1% SDS, 2% 2-mercaptoethanol, 5% glycerol). Gels were stained with silver.

Glycosidase treatment. For hydrolysis with peptide *N*-glycosidase F (glycopeptidase F; Boehringer Mannheim, Inc., Indianapolis, IN), 2 μg of purified azurocidin was first denatured by boiling in 0.125% SDS, 25 mM EDTA, 2.5% 2-mercaptoethanol, 100 mM $NaPO_4$, pH 8.0 for 3 min and the sample was brought to 1% Triton X-100. Glycopeptidase F was added at 60 mU/μl and the mixture was incubated at 37°C for 16 h. The sample was then boiled in SDS-PAGE sample buffer and subjected to electrophoresis as described above.

Isoelectric focusing. Isoelectric focusing was performed in 1-mm Ampholine PAGplates (pH range 3.5–9.5) on a Multiphor II electrophoresis unit (Pharmacia LKB Biotechnology, Piscataway, NJ) at 10°C at 30 mA, 30 W, 1,500 V, for 1.5 h. Gels were fixed in 10% TCA, 158 mM 5-sulfosalicylic acid, and processed for silver staining.

Microbicidal assays. Bactericidal activity was tested against *Escherichia coli* K12 (strain MC4100) and *Streptococcus faecalis* (ATCC 8043) as previously described (7). Fungicidal activity was tested against *Candida albicans* (clinical isolate from Columbia Presbyterian Hospital, New York) as previously described (9). Standard buffers used for the microbicidal assays were 50 mM sodium citrate, pH 5.5 (*E. coli,* 2 × 10^5 CFU/ml) and 10 mM sodium phosphate, pH 5.5 (*S. faecalis,* 2 × 10^5 CFU/ml, and *C. albicans,* 2 × 10^4 CFU/ml). Microbicidal activity was determined by calculating the decrease in colony forming units for microorganisms incubated with granule proteins as compared to control microorganisms incubated in buffer only. Typically, control colony forming units increased 10–20% from input colony forming units during the incubation at 37°C.

[^{3}H]DFP labeling of granule proteins. The incorporation of [^{3}H]-DFP (4.4 Ci/mmol; New England Nuclear, Boston, MA) followed the method of Young et al. (11) in a total volume of 80 μl with final concentrations of 100 mM Tris-HCl, pH 7.6, 50 nM [^{3}H]DFP and 15 μg/ml purified granule protein. After 1 or 16 h at 37°C, the reaction was terminated by the addition of SDS-PAGE sample buffer and boiled for 3 min. Half the sample was analyzed by SDS-PAGE on a 10% gel, stained with silver, soaked in EN^{3}HANCE (New England Nuclear) according to the manufacturer's recommendation, dried under vacuum, and subjected to autoradiography with Kodak X-OMAT AR film and an intensifying screen at −70°C for 14 d.

Proteolytic assays. Proteolysis of casein was assayed according to the method of Twining (12). For each sample, a 50-μl total volume consisting of 0.25% FITC-casein (type III, Sigma Chemical Co., St. Louis, MO), 50 mM Tris-HCl, pH 7.5 and 0.5–30 μg granule protein was incubated at 37°C for 12 h. The reaction was terminated by the addition of 120 μl of 5% TCA and allowed to precipitate on ice for 30 min. After centrifugation at 10,000 *g* for 15 min, 140 μl of TCA soluble supernatant was neutralized with 1.9 ml of 500 mM Tris, pH 8.5. Fluorescence was read in a Fluorolog spectrofluorometer (model 1680; Spex Industries, Edison, NJ) with an excitation wavelength of 490 nm and emission wavelength of 525 nm.

Proteolysis of elastin was assayed by a modification of the method of Banda et al. (13). A 300-μl reaction mix consisting of 0.5 mg fluorescein-elastin (200–400 mesh; ICN Biochemicals, Cleveland, OH), 50 mM Tris, pH 7.5 or 50 mM $NaPO_4$, pH 5.5 and 1–50 μg granule protein was incubated at 37°C for 18 h. The reaction was terminated by the addition of 800 μl of 500 mM Tris, pH 8.0, and immediate centrifugation of insoluble elastin at 10,000 *g* for 12 min. 1 ml of the supernatant was removed to a new tube containing 1 ml of 50 mM Tris, pH 7.5. Fluorescence was measured as in the casein assay.

Proteolysis of hemoglobin was assayed by the method of Kao et al. (5). The 100-μl reaction mix consisting of 1 mg hemoglobin, 10 mM Tris, pH 7.5 and 0.5–10 μg granule protein was incubated at 37°C for 16 h. Undegraded hemoglobin was precipitated with 800 μl of 5% TCA on ice for 60 min and pelleted at 10,000 *g* for 15 min. The absorbance at 280 nm of 800 μl of the supernatant was measured.

Nonspecific esterase activity was determined by an adaptation of the method of Barrett (14). The 100-μl reaction mix consisted of 50 mM Tris, pH 7.5, 50 μg/ml naphthol ASD acetate or α-naphthyl acetate (from a 5-mg/ml stock in DMSO) and 1–50 μg granule proteins. After incubation at 37°C for 5 h, 100 μl of coupling buffer was added, and the colored product was allowed to develop for 10 min before measuring the absorbance at 590 nm. The coupling buffer consisted of 0.3 mg/ml Fast Blue RR Salt, 2% Brij 35, 15 mM EDTA, and 100 mM KPO_4, pH 6.0.

A 96-well microtiter plate assay was adapted from the method of Castrillo et al. (15) to measure hydrolysis of synthetic tri- and tetrapeptide substrates coupled to chromogenic or fluorogenic groups (obtained from Bachem, Inc., Torrance, CA; Peninsula Laboratories Inc., Belmont, CA; and Sigma Chemical Co.). The amino acid sequences of the NH_2-terminal blocked substrates (*N*-succinyl- or *N-t*-butyloxycarbonyl-) are given in Table II. The substrates were solubilized at 20 mM in DMSO and diluted in DMSO to give a final DMSO concentration in the assay of 5%. The 220-μl reaction contained 50 mM $NaPO_4$, pH 7.5, 0.25–25 μg granule protein and 0.25 mM substrate for thiobenzyl ester and 7-amido-4-methyl coumarin derivatives or 0.5 mM substrate for 4-nitroanilide derivatives. Assays with thiobenzyl ester substrates also included 90 μM 5,5′-dithiobis 2-nitrobenzoic acid. The plates were incubated at 37°C for 90 min. Chromogenic assays were monitored spectrophotometrically with a 405-nm filter in a plate reader (model EL-307; Biotek Instruments, Burlington, VT). Fluorogenic assays were monitored in a microplate fluorescence reader (Fluoroskan I; Flow Laboratories, McLean, VA).

Production of anti-azurocidin and anti-p29b antibodies. Rabbit antisera were produced by intradermal injection of New Zealand White rabbits with 50 μg of azurocidin or p29b purified as described and emulsified with Freund's complete adjuvant. Three subsequent immunizations were made at 3-wk intervals with 50 μg azurocidin or p29b in Freund's incomplete adjuvant. Serum was obtained 9 d after the fourth immunization. Preimmune and immune immunoglobulins were purified by affinity chromatography on Protein A Sepharose (Pharmacia LKB Biotechnology) according to the manufacturer's recommendations and stored at 7 mg/ml in PBS at −70°C.

Immunoblotting. Proteins were separated by SDS-PAGE and transferred to nitrocellulose by the procedure of Towbin et al. (16) with modifications to improve the transfer of cationic proteins. After electrophoresis, the gel was equilibrated in transfer buffer: 50 mM Tris-base, 40 mM glycine, pH adjusted to 8.3 with HCl, 0.1% SDS, 20% methanol. The transfer to nitrocellulose was conducted in a Hoeffer Transphor Unit (Hoeffer Scientific Instruments, San Francisco, CA) at room temperature at 70 V for 2 h. The nitrocellulose blots were blocked with 5% nonfat dry milk, 0.05% Tween 20, PBS, pH 7.4 overnight at 4°C. Blots were incubated with antibodies diluted in the blocking solution for 1 h at room temperature followed by 16 h at 4°C. After washing with 0.05% Tween 20 in PBS the blots were reacted with affinity purified goat IgG anti-rabbit IgG (heavy and light chains) conjugated to alkaline phosphatase (Boehringer Mannheim). Immunoreactive bands were visualized by incubation with the substrate: 0.5 mg/ml nitro blue tetrazolium, 0.1 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, 4 mM $MgCl_2$ in veronal-acetate buffer (142 mM barbital, 143 mM sodium acetate, pH 9.5).

Indirect immunofluorescence. PMN isolated as above were sedimented onto glass slides by cytocentrifugation (Shandon, Inc., Sewickley, PA), fixed 20 min at room temperature in 2% paraformaldehyde, 10 mM $NaPO_4$, pH 7.5, 100 mM lysine, 60 mM sucrose, 14 mM

sodium *m*-periodate. The cells were rinsed in PBS, permeablized in acetone at −20°C for 5 min, rinsed in PBS and blocked with 10% FCS in PBS for 20 min. Specific antibodies or preimmune antibody diluted in 1% FCS in PBS were added for 90 min in a humidified chamber at room temperature. Slides were washed in PBS, incubated with rhodamine isothiocyanate-conjugated goat IgG anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in 1% FCS/PBS for 45 min, washed in PBS and mounted with glycerol/PBS (9:1). Immunoreactivity was observed in a Nikon fluorescence microscope with a G (546 nm) filter and photographed with Ektachrome 400 or T-MAX 400 film (Eastman Kodak, Rochester, NY).

Immunoelectron microscopy. PMN (4×10^7 cells) were suspended in 2% paraformaldehyde (E.M. grade; E.M.S., Fort Washington, PA), 0.05% glutaraldehyde (E.M. grade, E.M.S.) in PBS for 90 min on ice. The cells were washed twice in PBS and embedded in agar (Becton Dickinson, & Co., Cockeysville, MD) before dehydration in a graded series of solutions of ethanol and water and infiltration/embedding in Lowicryl K4M (Ted Pella, Inc., Tustin, CA) at −25°C as directed by the manufacturer. Polymerized blocks were sectioned on an MT6000-XL ultramicrotome (RMC, Inc., Tucson, AZ) and 80-nm sections were picked up on nickel grids coated with formvar and carbon. Sections were incubated sequentially in blocking buffer (1% BSA, 1% gelatin, 150 mM NaCl, 20 mM Tris, pH 8.4) for 5 min at 25°C, antibody diluted in blocking buffer for 1 h at 25°C and 16 h at 4°C and protein A colloidal gold (10 nm) diluted to an OD_{520} of 0.060 in blocking buffer for 1 h at 25°C with extensive washing in 150 mM NaCl, 20 mM Tris, pH 8.4 before and after the incubation with colloidal gold. Grids were rinsed in distilled H_2O and thoroughly air dried before staining with uranyl acetate and lead citrate. Samples were viewed on a JEOL 100CX electron microscope and photographed at 80 KV. Antibodies were titered for specific labeling and the following dilutions were chosen: preimmune and anti-azurocidin 1:8000, anti-lactoferrin (2.6 mg/ml, affinity purified, Jackson ImmunoResearch Laboratories, Inc.) 1:400, anti-cathepsin G (rabbit serum, a gift of I. Olsson, University of Lund, Sweden) 1:20,000. Controls in addition to preimmune IgG included labeling with protein A gold alone in the absence of antibody, preabsorbing anti-lactoferrin with a 200-fold molar excess of lactoferrin (Jackson ImmunoResearch Laboratories, Inc.) coupled to Sepharose, or preabsorbing anti-azurocidin with a 30 to 300-fold molar excess of azurocidin purified as described above. Each of these procedures reduced labeling by 80–90% (results not shown).

For each labeling condition at least 30 micrographs were taken at a magnification of 20,000 of at least 20 cell profiles. Gold particles over the total cellular area and within each subcellular compartment (nucleus, azurophil granules, and specific granules) were counted directly from the negatives. Azurophil granules were identified morphologically as large, electron-lucent structures that labeled positively with anti-cathepsin G. Specific granules were identified as smaller, gray granules that labeled with anti-lactoferrin. The cytoplasmic compartment was defined as any non-nuclear, non-granular area and the number of gold particles in this compartment was obtained by subtracting nuclear and granular particles from the total particles on each negative. The areas of nucleus, azurophil, and specific granules were measured and summed for each negative using the Microcomp Planar Morphometry program (Southern Micro Instruments, Atlanta, GA). Cytoplasmic area was obtained by subtracting nuclear and granular areas from the total cellular area in each micrograph.

Suborganelle localization. Azurophil granules from 10^8 cells were lysed with seven rounds of freeze-thaw-sonication followed by centrifugation at 100,000 *g* for 20 min at 4°C. The supernatant was recentrifuged at 100,000 *g* for 20 min to obtain the soluble granule contents (granulosol). The pelleted granule membrane associated material (granule membranes) was washed twice by resuspension in 100 mM $NaPO_4$, pH 7.0, and centrifugation at 100,000 *g* for 20 min at 4°C. Total granules, granule membranes, and granulosol were extracted with either acid (50 mM glycine, pH 2.0) or salt (2 M NaCl, 50 mM $NaPO_4$, pH 7.0) for 40 min at 20°C followed by centrifugation at 100,000 *g* for 20 min at 4°C. The resulting supernatants, containing proteins solubilized by the two extraction methods, were analyzed on a Vydac C4 reverse phase HPLC column as described above. Relative protein yields were compared by weighing the peaks cut from the chart recording of the absorbance at 214 nm.

Results

Purification and biochemical characterization of azurocidin and p29b. Acid extracts of PMN azurophil granule membrane associated material (for simplicity, hereafter referred to as granule membranes) were subjected to HPLC gel filtration, and the column fractions were analyzed by SDS-PAGE (Fig. 1). Proteins of 25-29 kD and 4 kD eluted in peaks B and C while an 80-kD protein eluted in peak A. The gel filtration fractions were screened for antimicrobial activity against *E. coli* and *C. albicans.* As previously reported, potent microbicidal activity was found in peaks B and C but only trace activity was associated with peak A (data not shown; see Fig. 1 of reference 9).

The components of gel filtration peak C were further puri-

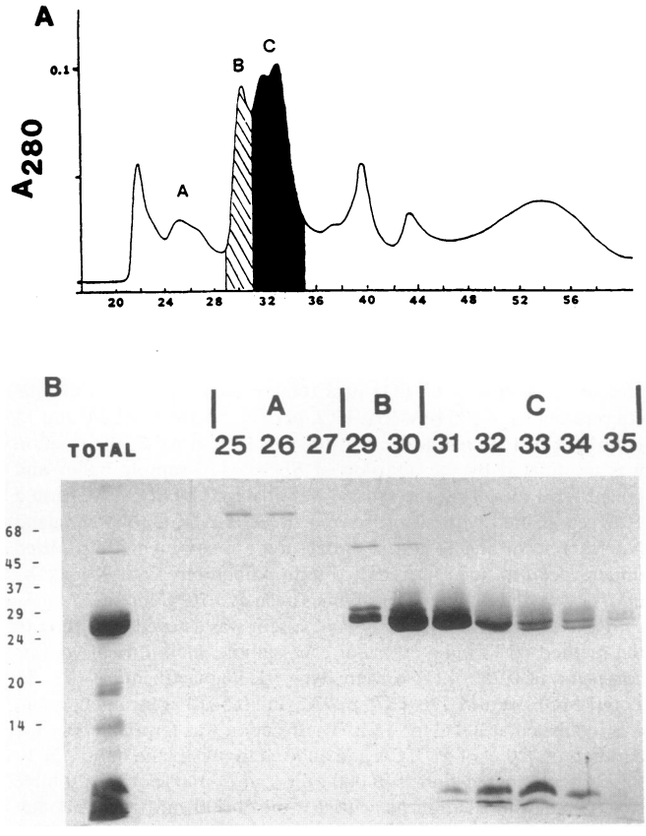


Figure 1. HPLC size-exclusion chromatography of azurophil granule membrane extract. (*A*) Profile of the absorbance at 280 nm. Fraction numbers are indicated on the abscissa. (*B*) Gel filtration fractions and the acid extract of the azurophil granule membranes (total) were resolved on 15% reducing gels stained with silver. Fractions were pooled into peaks *A*, *B*, and *C* as indicated. Migration of molecular mass standards in kilodaltons is indicated.

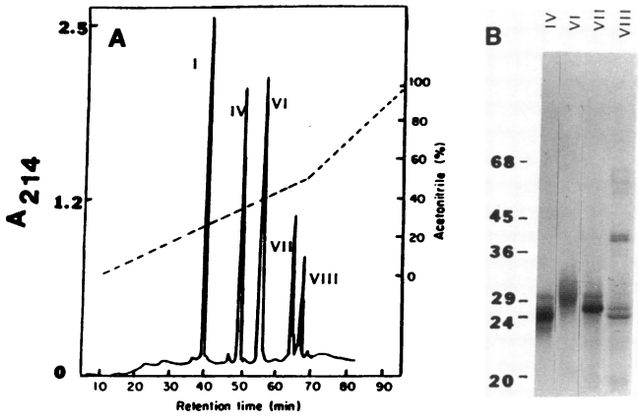


Figure 2. Reverse phase HPLC purification of azurocidin from gel filtration fractions. (*A*) The absorbance at 214 nm of peak C of the chromatogram in Fig. 1 eluted from a C4 reverse-phase column with a gradient of acetonitrile. (*B*) SDS-PAGE analysis of the reverse phase HPLC peaks on a 10% reducing gel stained with silver. IV, cathepsin G; VI, azurocidin; VII, p29b; VIII, elastase (peaks numbered according to ref. 6). Peak I (defensins; HNP 1–3) is not shown. Migration of molecular mass standards in kilodaltons is indicated.

fied by reverse-phase HPLC, yielding 5 major peaks (Fig. 2 *A*) that were identified by comparison to the elution profile obtained when extracts of azurophil granule membranes were directly chromatographed by reverse phase HPLC (6). Peak VI was comprised of a protein migrating diffusely at 29 kD on SDS-PAGE (Fig. 2 *B*). Its identity with azurocidin was confirmed by amino terminus sequencing (Scott, R., D. Campanelli, C. Nathan, and J. Gabay, unpublished result). Also purified in the reverse-phase fractionation of gel filtration peak C were defensins (Fig. 2 *A*, peak I), cathepsin G (peak IV), p29b (peak VII), and elastase (peak VIII).

The silver stained gel in Fig. 2 *B* illustrates the purity of azurocidin and p29b. The 80-kD species that contaminated earlier preparations of azurocidin was absent. Azurocidin appeared as a diffuse band, but when less protein was electrophoresed, at least four individual bands could be resolved (not shown). Consistent with the possibility that these bands represent glycosylation isoforms was the finding that azurocidin displayed an increased electrophoretic mobility and fewer discrete bands on SDS-PAGE after treatment with peptide *N*-glycosidase F (not shown).

Azurocidin and p29b migrated completely to the cathode in polyacrylamide isoelectric focusing gels of pH range 3.5 to 9.5. The strongly cationic cathepsin G (pI > 12.5) (17) displayed a similar migration pattern (data not shown).

Antimicrobial activity. The antimicrobial activities of azurocidin and p29b were reported using preparations of azurocidin contaminated with an 80-kD protein (6). The antimicrobial activity of azurocidin was retested against three mi-

Table I. Antimicrobial Activity of Azurocidin, p29b, and Cathepsin G

	Yield	LD_{50}		
	μg/10⁸ cells	*E. coli*	*μg/ml S. faecalis*	*C. albicans*
Azurocidin	24	0.5±0.1 (10)*	1.5±0.2 (9)	5.7±0.4 (8)
p29b	10	0.4±0.1 (3)	1.4±0.3 (4)	13.4±2.6 (3)
Cathepsin G	26	2.7±1.1 (4)	0.2±0.1 (5)	0.9±0.1 (6)

* The LD_{50} was determined from dose-response curves of the type illustrated in Fig. 3. Mean±SEM of the number of experiments in parentheses.

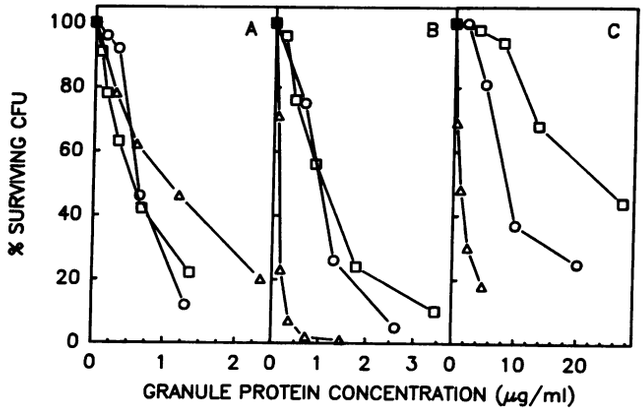


Figure 3. Dependence of antimicrobial activity on granule protein concentration. Microbicidal assays were conducted in the standard buffers with azurocidin (○), p29b (△), or cathepsin G (□) against three microorganisms: (*A*) *Escherichia coli;* (*B*) *Streptococcus faecalis;* (*C*) *Candida albicans.*

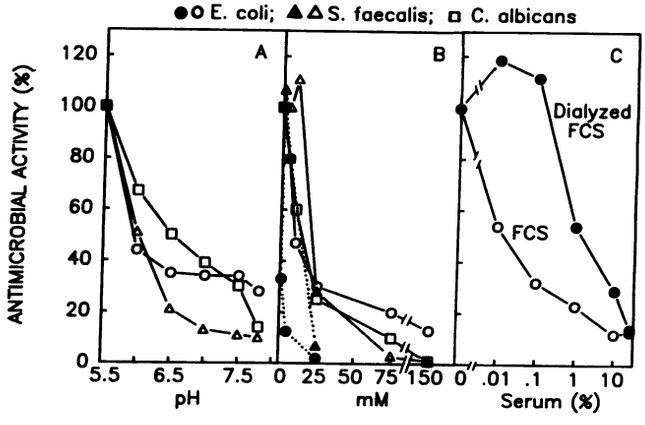


Figure 4. Dependence of the antimicrobial activity of azurocidin on physiologic conditions. (*A*) Effect of pH. Antimicrobial activity against all three organisms was assayed in 10 mM $NaPO_4$, pH 5.5 to 7.8 and is presented as a percentage of the activity found in pH 5.5 buffer. (*B*) Effect of NaCl and $CaCl_2$. Antimicrobial activity was assayed as in Fig. 3 with the addition of NaCl (*solid line*) or $CaCl_2$ (*dotted line*) and is presented as a percentage of the activity found with no additional salts (0 mM). (*C*) Effect of serum. Antimicrobial activity against *E. coli* was assayed in 50 mM citrate, pH 7.0. Heat inactivated FCS, with or without prior dialysis against the assay buffer, was added to a final concentration ranging from 0.01 to 25%. The antimicrobial activity is presented as a percentage of the activity found with no added serum (0%, LD_{50} = 1.1 μg/ml). All data points are the average of two experiments.

Table II. Proteolytic Activity of Azurophil Granule Proteins

Substrate	Enzymes			
	Cathepsin G	Azurocidin	p29b	Elastase
Proteins				
Casein	2180*	0	14580	11500
Elastin pH 7.5	6360*	38	4180	10400
Elastin pH 5.5	1220*	0	2580	3710
Hemoglobin	0.020‡	0.001	0.097	0.616
Organic esters				
Naphthol ASD Acetate	0.836§	0.004	0.012	0.411
α-Naphthyl Acetate	0.418§	0.002	0.015	0.526
Peptides‖				
Ala Ala Pro Phe SBzE¶	0.250**	0.001	0.012	0.001
Phe Pro Phe 4NA¶	0.183**	0.002	0.000	0.021
Ala Ala Phe AMC¶	42.4*	0	0	0
Leu Leu Val Tyr AMC¶	62.4*	0	0	0
Ala Ala Pro Val AMC‡‡	57.6*	0	3.9	287
Ala Pro Ala AMC‡‡	0*	0	2.0	26.8
Gly Gly Arg AMC§§	0*	0	0	0
Phe Ser Arg AMC§§	1.0*	0	0	0
Val Leu Lys AMC§§	4.0*	0	0	0
Val Pro Arg AMC§§	4.8*	0	0	0
Gly Pro Leu Gly Pro AMC‖‖	0*	0	0	0

Purified granule proteins in a range of equal molar concentrations (see Methods) were tested against the substrates listed. Results are shown for that concentration of granule protein giving a response in the midpoint of the linear phase of the concentration-response curve, or if no response was seen, for the highest concentration tested. Results are normalized per milligram granule protein and are the mean of duplicates. The experiment shown was repeated once with similar results.

* Fluorescence/mg/ml/h.

‡ OD_{280}/mg/ml/h.

§ OD_{590}/mg/ml/h.

‖ *Abbreviations:* SBzE, thiobenzyl ester; 4NA, 4-nitroanilide: AMC, 7-amido-4-methyl-coumarin.

¶ Substrate for chymotrypsin-like proteases.

** OD_{405}/mg/ml/h.

‡‡ Substrate for elastase-like proteases.

§§ Substrate for trypsin-like proteases.

‖‖ Substrate for collagenase-like proteases.

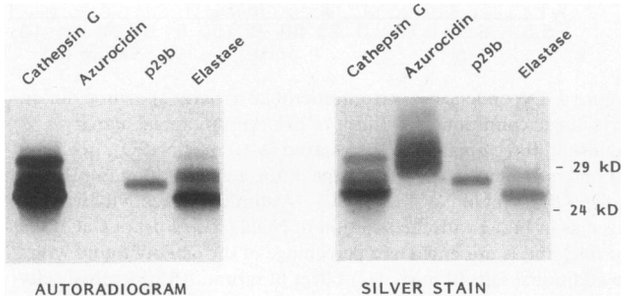


Figure 5. [^{3}H]DFP labeling and SDS-PAGE analysis of azurocidin and p29b. Purified proteins were incubated with [^{3}H]DFP, resolved on 10% gels under reducing conditions, stained with silver and visualized by autoradiography. Migration of molecular mass standards in kilodaltons is indicated.

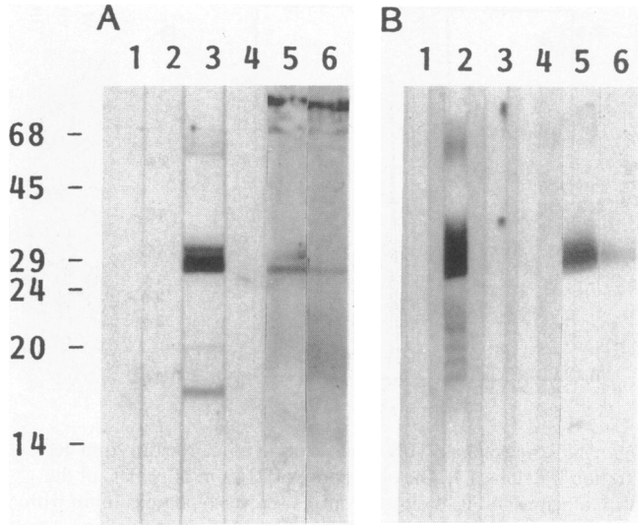


Figure 6. Immunoblot analysis of purified granule proteins and granule extracts with anti-azurocidin and anti-p29b antibodies. 0.5 μg of cathepsin G, azurocidin, p29b or elastase (lanes *1–4*, respectively) or 5 μg of acid extracts of azurophil granules (lane *5*) and specific granules (lane *6*) were subjected to SDS–PAGE on 15% gels, transferred to nitrocellulose and immunoblotted with either anti-p29b IgG (*A*) or anti-azurocidin IgG (*B*). Migration of molecular mass standards is indicated.

croorganisms using preparations free of the 80-kD protein. For comparison, p29b and cathepsin G purified by the same procedure were tested under the same conditions. The representative experiments in Fig. 3 illustrate the concentration dependence of antimicrobial activity. LD_{50}'s compiled from several such experiments are presented in Table I. On average, azurocidin exhibited > 5 times the activity of cathepsin G against *E. coli* but only $^1/_5$ to $^1/_{10}$ the activity of cathepsin G against *S. faecalis* and *C. albicans*. p29b was as potent as azurocidin against *E. coli* and *S. faecalis* but half as potent against *C. albicans*. Azurocidin and p29b had similar potency when purified by gel filtration and reverse phase HPLC or when prepared by reverse phase HPLC alone (6).[2]

Experiments to characterize the effects of varying physiologic conditions on antimicrobial activity focussed on azurocidin. Killing was detectable over the entire pH range tested (pH 5.5–7.8) (Fig. 4 *A*). Activity against each of the target organisms was enhanced by mildly acidic conditions.

The effect of osmotic and ionic strength on the antimicrobial activity of azurocidin at pH 5.5 was studied by the addition of sucrose, NaCl, or $CaCl_2$. The addition of sucrose up to a final concentration of 600 mosM had no effect on the microbicidal activity (data not shown). In contrast, the addition of NaCl or $CaCl_2$ markedly reduced the activity against all three organisms (Fig. 4 *B*). For *E. coli* an 80% reduction of activity was seen with the addition of 75 mM NaCl. $CaCl_2$ had

2. An apparent discrepancy in LD_{50}s of p29b for *Streptococcus faecalis* is due to a calculation error in the data presented in reference 6, Table II, where the LD_{50} for peak VII against *S. faecalis* should read 5.3 μg/ml.

an even greater inhibitory effect; 10 mM $CaCl_2$ resulted in an 80% reduction of killing of *E. coli.*

To examine other effects of the extracellular milieu on antimicrobial activity, assays were performed in serum at pH 7.0 (Fig. 4 *C*). The presence of heat-inactivated FCS during the assay had a dose-dependent inhibitory effect above a final concentration of 0.01%. An 80% reduction in killing of *E. coli* occurred in the presence of 10% serum. In experiments designed to control for the effect of ionic strength, serum was first dialyzed extensively against the microbicidal assay buffer (50 mM citrate, pH 7.0) until all concentrations of serum attained the same osmolarity. Dialyzed serum was inhibitory, but nearly 100-fold less so than equal concentrations of nondialyzed serum. Results were similar with fetal calf plasma (data not shown). Bovine serum albumin was not inhibitory up to the concentrations of albumin found in 25% serum (12 mg/ml, data not shown).

Proteolytic activity. The amino terminal sequences for azurocidin and p29b have recently been reported (6). Sequences of the first 20 amino acids of these two proteins were

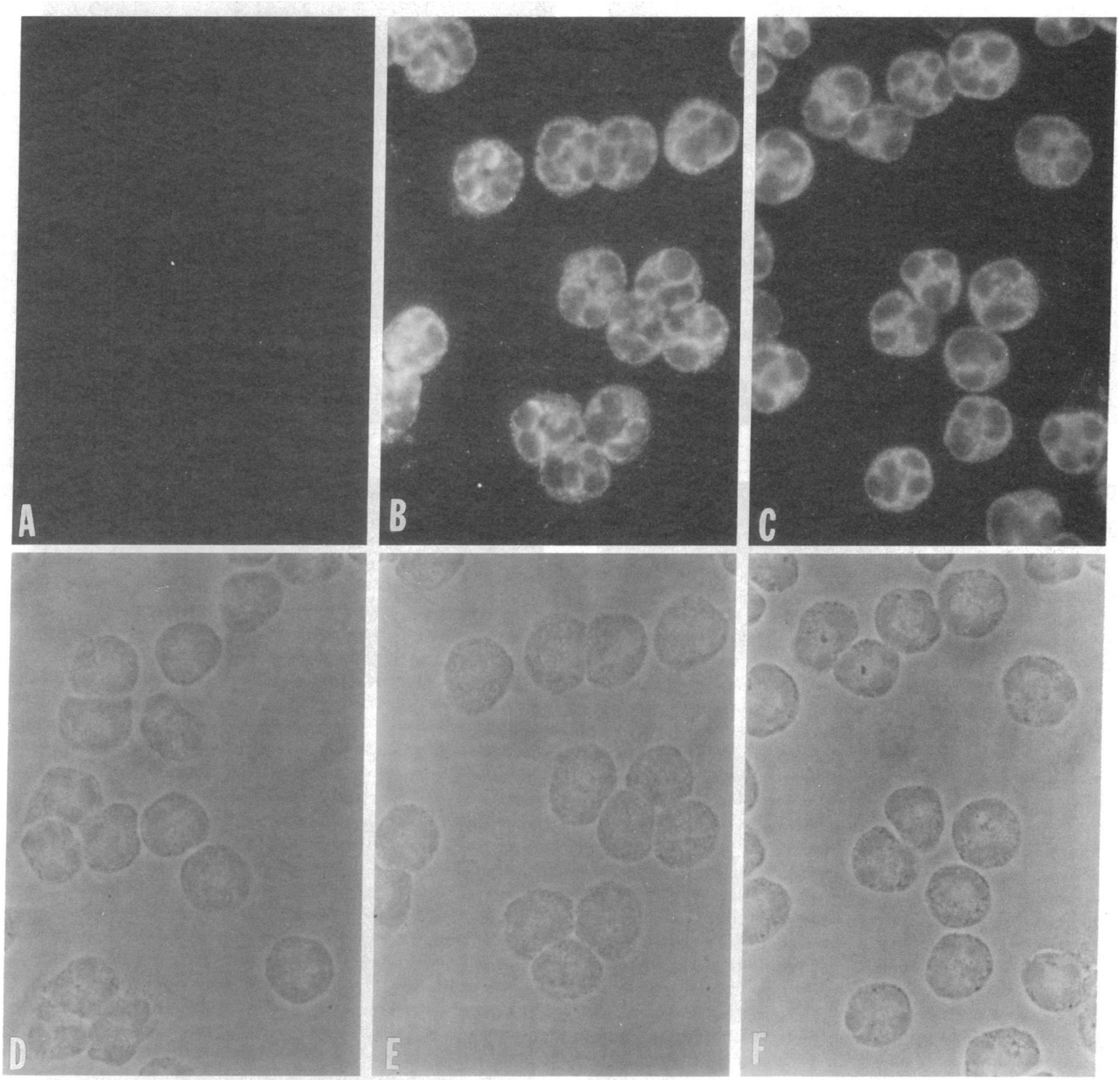
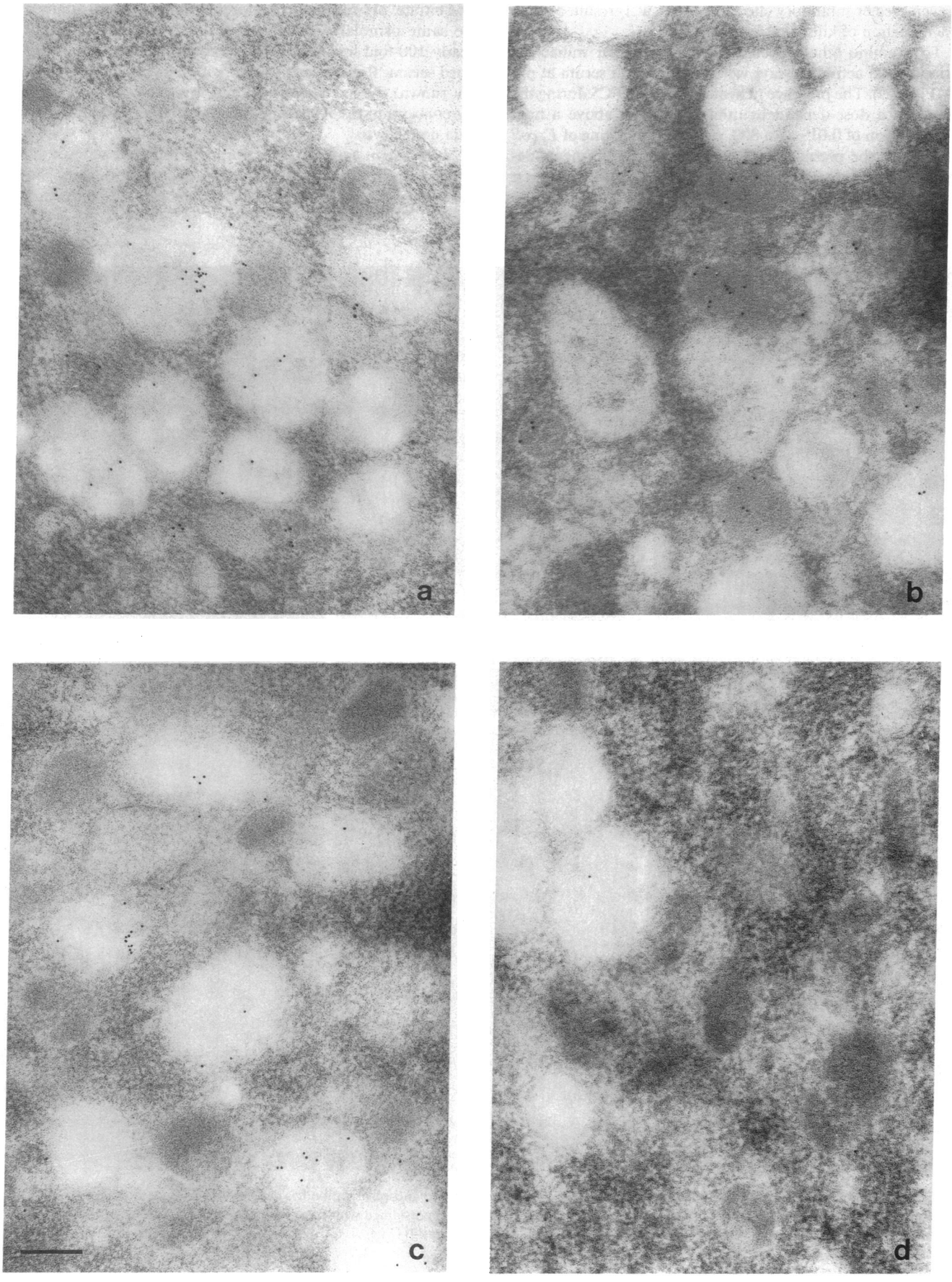


Figure 7. Immunofluorescence labeling of neutrophils with anti-azurocidin and anti-p29a antibodies demonstrates a punctate cytoplasmic distribution. PMN were cytocentrifuged, fixed with paraformaldehyde/acetone, and stained with preimmune IgG (*A, D*), anti-azurocidin (*B, E*), or anti-p29b (*C, F*). Indirect immunofluorescence with rhodamine conjugated anti-IgG (*A, B, C*). The corresponding phase contrast micrographs are shown below (*D, E, F*). (×1,000).


a
b
c
d

50 to 65% identical with the N-terminal sequences of both cathepsin G and elastase. Moreover, many of the differences between azurocidin, p29b and the other two granule proteins represented conservative changes that could be accounted for by single base pair mutations. Based on this amino terminal homology, azurocidin, and p29b appear to be members of the serine protease family.

The neutral esterase activity of azurocidin and p29b was investigated by assaying the hydrolysis of protein, organic ester, and synthetic peptide substrates, as well as by radiolabeling with a serine esterase-specific inhibitor. As positive controls, cathepsin G and elastase purified under the same conditions as azurocidin and p29b were included in all assays (Table II). Experiments with the substrates casein, hemoglobin and elastin revealed significant proteolytic activity of p29b. In contrast, protease activity of azurocidin with each of these protein substrates was less than 1% the activity of cathepsin G, p29b, or elastase. Assays with elastin as a substrate were performed at the neutral pH required by serine proteases, as well as at pH 5.5, the condition necessary for activity of acid/aspartic proteases. The activity of p29b was reduced by only 38% at pH 5.5, while the activities of cathepsin G and elastase were reduced more dramatically at the lower pH (81% and 65%, respectively). No proteolytic activity of azurocidin was detectable under acidic conditions.

The nonspecific esterase substrates naphthol ASD acetate and α-naphthyl acetate were hydrolyzed weakly by p29b (3 to 13% of the specific activity of elastase and cathepsin G). The nonspecific esterase activity of azurocidin was only 0.5 to 2% that of cathepsin G or elastase. Finally, we tested synthetic peptide substrates that have been well characterized in assays for trypsin-like, chymotrypsin-like, elastase-like, and collagenase-like proteases. Cathepsin G and elastase effectively hydrolyzed their predicted substrates (Table II). No significant cleavage of any of the peptide substrates by azurocidin could be detected under the conditions of the assays. p29b was inactive against most of the peptide substrates that were cleaved by cathepsin G and elastase, and only weakly hydrolyzed the substrates Ala-Ala-Pro-Phe, Ala-Ala-Pro-Val, and Ala-Pro-Ala (1–7% of the activity of cathepsin G or elastase). This result argues strongly against the possibility that the high proteolytic activity of p29b on protein substrates is due to contamination by elastase or cathepsin G.

Since most serine proteases bind and are irreversibly inhibited by DFP, radiolabeling of the purified granule proteins with tritiated DFP was attempted. Labeled proteins were analyzed by SDS-PAGE and detected by autoradiography. The autoradiograph in Fig. 5 demonstrates the complete absence of labeling of azurocidin under conditions that labeled equal amounts of cathepsin G, p29b, and elastase. Therefore, p29b is a serine protease. In contrast, neither protease, peptidase, nor esterase activity, nor binding of the serine protease inhibitor DFP could be attributed to azurocidin.

Cellular and subcellular localization. Polyclonal antibodies raised against purified azurocidin and p29b were used for immunoblotting, immunofluorescence and immunoelectron microscopy to identify the cellular and subcellular localization of these proteins. To assess the specificity of the antibodies, equal amounts of cathepsin G, elastase, p29b, and azurocidin were immunoblotted with anti-azurocidin or anti-p29b antibody (Fig. 6). The antibodies reacted only with their respective antigens, despite the sequence homology among the four proteins. Faintly reactive bands in the 68-kD region of all lanes were also observed with preimmune IgG (not shown). Immunoreactive bands of lower molecular mass (14–20 kD) were inconsistently detected and may represent degradation products of the 29-kD proteins. When fractions from Percoll density gradients were immunoblotted with either the anti-azurocidin or anti-p29b antibodies, those fractions enriched in azurophil granules displayed strongly reactive 29-kD bands, while specific granule-enriched fractions were only weakly reactive with the antibodies (Fig. 6). By densitometric scanning, the staining of azurophil granule extracts was 10-fold more intense with anti-azurocidin and 4-fold more intense with anti-p29b than the staining of specific granule extracts (data not shown). No reactivity was observed in the plasma membrane or cytosolic fractions (data not shown). The weak staining of specific granule extracts by the anti-azurocidin antibody may reflect the contamination of the specific granule preparation with azurophil granules, as evidenced by its content of 5–10% of the recovered myeloperoxidase and β-glucuronidase, two markers of the azurophil granule (data not shown, see reference 7). The immunoblotting of specific granules by anti-p29b was more intense than could be accounted for by azurophil granule contamination, leaving open the possibility that an immunologically related protein may exist in specific granules.

Indirect immunofluorescent labeling of permeablized neutrophils with the anti-azurocidin and anti-p29b antibodies gave strong positive fluorescence in a granular pattern in the cytoplasm (Fig. 7). Preimmune antibody at the same concentration did not stain.

Immunoelectron microscopy was used to refine the subcellular localization of azurocidin. When sections of Lowicryl-embedded neutrophils were incubated with anti-azurocidin antibody and protein A-gold, labeling was observed predominantly over large, electron-lucent granules (Fig. 8 *a*). These granules were also labeled by anti-cathepsin G antibody (Fig. 8 *c*) and were therefore identified as azurophil granules. In contrast, anti-lactoferrin antibody labeled the smaller, electron-dense specific granules (Fig. 8 *b*). The density of gold particles labeling the nucleus, cytoplasm, azurophil granules and specific granules was quantitated for each of the antibodies (Table III). The anti-azurocidin antibody specifically labeled the azurophil granules. The density of label was 8–10-fold higher than that found in the nucleus, cytoplasm, or specific granules and closely paralleled the distribution of label for the anti-cathepsin G antibody. Preincubation of the anti-azurocidin antibody with purified azurocidin before incubation with the neutrophil sections abolished the immunolabeling (data not shown).

Figure 8. Immunoelectron microscopy of neutrophils labeled with anti-azurocidin and protein A gold. Labeling with anti-azurocidin (*a*) is distributed predominantly over the larger, electron-lucent azurophil granules in a pattern similar to that seen with anti-cathepsin G (*c*). Anti-lactoferrin (*b*) labeled the smaller, electron-dense specific granules. (*d*) Preimmune IgG. ×56,000. Bar = 0.2 μm.

Table III. Labeling of Azurophil Granules by Anti-azurocidin

Antibody	Density of label (particles/μm^2)			
	Azurophil granules	Specific granules	Nucleus	Cytoplasm
Azurocidin	42.4±15.1	5.3±4.6	5.9±3.8	4.2±3.4
Cathepsin G	38.4±15.4	2.4±1.8	4.8±2.6	4.0±2.2
Lactoferrin	2.6±1.6	132±38	1.9±1.6	4.9±3.4
Preimmune	1.3±1.5	2.5±6.0	0.6±0.7	0.6±0.6

Values are the mean±SEM. Sections of Lowicryl-embedded PMN were labeled with polyclonal IgG and protein A colloidal gold. The density of label over the four cellular compartments was determined as described in Methods.

To further define the location of azurocidin within the azurophil granules, the granules were fractionated into granule membrane associated material (granule membranes) and soluble granule contents (granulosol). Total granules, granule membranes or granulosol were extracted with acid and the extracts were analyzed by the C4 reverse-phase chromatography procedure used in the purification of azurocidin. Quantification of the relative yields of azurocidin and three representative granule proteins are presented in Table IV. The majority of azurocidin (> 88%), cathepsin G (> 94%) and BPI (> 83%) partitioned with the granule membrane, while 96% of lysozyme was released into the granulosol after granule lysis.

The property of resistance of integral membrane proteins to solubilization by high salt buffers was used to characterize the association of azurocidin with the granule membrane. Azurophil granule membranes were extracted with either high salt buffer (2 M NaCl, 50 mM $NaPO_4$, pH 7.0) or acid (50 mM glycine, pH 2.0) and subjected to reverse-phase HPLC for comparison of protein yields (Table IV). Salt was capable of solubilizing 84% of the azurocidin and 100% of the cathepsin G that was extractable by acid. In contrast, salt treatment could solubilize only 17% of the BPI that was extractable in acid. Solubilization with high salt buffers implies that azurocidin is not an integral membrane protein.

Table IV. Suborganelle Localization of Azurocidin and Three Other Azurophil Proteins

Compartment	Azurocidin		Cathepsin G		Lysozyme		BPI	
	H^+*	NaCl‡	H^+	NaCl	H^+	NaCl	H^+	NaCl
Total granule	367§	313	372	385	260	226	30	6
Granulosol	55	66	28	23	238	237	4	3
Membrane	392	331	416	422	10	2	17	3

Azurophil granules (total granule) were separated into soluble granule contents (granulosol) and membrane associated material (membrane). Each compartment was then extracted with acid (H^+) or high salt buffer (NaCl) and analyzed by reverse-phase HPLC as described in Methods.

* 50 mM glycine, pH 2.0.

‡ 2 M NaCl, 50 mM $NaPO_4$, pH 7.0.

§ Relative units.

Discussion

Human neutrophils contain within their azurophil granules at least four members of the neutral serine protease family, based on NH_2-terminal sequence homology: elastase, cathepsin G, p29b, and azurocidin. These differ in their relative proteolytic and antimicrobial activities.

p29b bound DFP, a label specific for the serine protease catalytic site (18), and hydrolyzed elastin, casein and hemoglobin; no synthetic peptide substrate could be identified. The proteolytic activity profile of p29b resembles that of proteinase 3, originally described by Dewald et al. as an α-naphthyl acetate esterase (19) and more recently purified and characterized as an elastinolytic serine esterase capable of inducing emphysema in hamsters (5, 20). Like p29b, the 29-kD cationic proteinase 3 is inactive against elastase-specific or cathepsin G-specific synthetic substrates. Positive identification of p29b with proteinase 3 awaits publication of NH_2-terminal amino acid sequence of the latter.

Azurocidin did not bind DFP and was inactive against all of the protease substrates tested. Therefore, its classification in the serine protease family is based solely on the high degree of homology of the NH_2-terminal sequences. We cannot exclude the possibilities that azurocidin has a highly restricted substrate specificity, requires cofactors or assay conditions not typically associated with serine proteases, or only becomes proteolytic after activation. For example, plasminogen activator, a serine protease of the specific granules of neutrophils, is stored in an amidolytically inactive proenzyme form that cannot bind DFP (21). The serine esterases of the granules of murine cytotoxic T lymphocytes, called "granzymes" (22), include at least six distinct polypeptides with highly homologous NH_2-termini and variable reactivity with esterase substrates. Like azurocidin, two of the granzymes are unable to bind DFP. Their function is unknown; the possibility that they have antimicrobial activity has apparently not been tested.

Azurocidin and p29b showed greatest antimicrobial activity against the gram-negative bacterium *E. coli*. Killing could also be detected against the gram-positive bacterium *S. faecalis* and the fungus *C. albicans*. In the latter case, azurocidin was slightly more potent than p29b. Additional target microorganisms must be tested to compare the spectra of activity and relative potencies of these two proteins. Various bacteria differ in their relative susceptibility to cathepsin G (23), bactericidal/permeability-increasing protein (BPI) (24), cationic antimicrobial proteins (CAP 57 and CAP 37) (25), and defensins (26), and a similar situation may pertain for azurocidin or p29b. Acidic conditions (pH 5.5), as proposed to prevail in the mature phagolysosome (27), resulted in enhanced activity of azurocidin and p29b against all three organisms tested. An acid pH optimum against gram-negative bacteria has been reported for CAP 37 (28). The antimicrobial activity of azurocidin and p29b is insensitive to DFP (6), implying a nonproteolytic mechanism of antimicrobial action, as for cathepsin G (3, 23).

Ionic solutes interfered with the microbicidal action of azurocidin, possibly by inhibiting its binding to targets, as proposed for cathepsin G (29). The initial attachment of BPI to bacteria is mediated principally by a calcium- and magnesium-sensitive electrostatic interaction between the cationic BPI and anionic sites on lipopolysaccharide (30). Little experi-

mental evidence exists concerning the ionic composition of the phagolysosome of PMN. Studies in human macrophages predicted an intraphagolysosomal calcium concentration below 100 μM (31). This level is well below the 1–5 mM calcium concentration found to inhibit azurocidin in the in vitro microbicidal assay. The sensitivity to isotonic NaCl reported for many azurophil granule antimicrobial proteins tested (3, 6, 26, 28), including azurocidin, suggests not only limited action extracellularly, but also suboptimal activity within the apparently "unsealed" phagolysosomal vacuoles that are in communication with the extracellular space (32).

The inhibitory effects of serum and plasma on antimicrobial activity could mostly be accounted for by ionic strength, but nondialyzable components were also implicated. α_1-antiprotease, α_1-antichymotrypsin, and α_2-macroglobulin bind and inhibit elastase and cathepsin G (33, 34). The interaction of these serum protease inhibitors with azurocidin and p29b remains to be explored. Bovine serum albumin inhibits the bactericidal action of BPI without preventing BPI's binding to bacteria (35). Albumin is not responsible for the inhibitory effect of serum on azurocidin.

Although azurocidin and p29b were purified from a relatively homogeneous preparation of azurophil granules (7), it was necessary to confirm the specificity of their subcellular localization. The pattern of immunofluorescent staining with antibodies against these two proteins was consistent with a granular location. Localization to the azurophil granules by immunoblot analysis of subcellular fractions was further confirmed for azurocidin by immunoelectron microscopy. Storage within azurophil granules should permit these antimicrobial agents to be delivered to their presumed site of action within the phagolysosome. The nonintegral association of azurocidin with the granule membrane associated material is not yet understood, but could result from ionic interactions between this basic protein and the acidic proteoglycans that comprise the granule matrix, as has been proposed for the serine esterase and carboxypeptidase of the secretory granules of natural killer cells (36). In contrast, BPI, which is not extracted from granule membranes with high salt buffers, may be an integral membrane protein (37, 38).

Serine proteases have been characterized in most types of lymphohematopoietic cells, including PMN (1), mast cells (39), macrophages (40), cytolytic T lymphocytes (22), and lymphokine activated killer cells (41). It is likely that these serine proteases have arisen by gene duplication and mutation (42), creating proteins with precise substrate specificity for narrowly defined proteolytic functions. Antimicrobial function of these granule proteins may have evolved separately from protease activity, generating polypeptides with a complex spectrum of antimicrobial and proteolytic activities.

Attention has focused on the release of neutral serine proteases from PMN in the pathogenesis of emphysema (1, 5), glomerulonephritis (43, 44), and adult respiratory distress syndrome (45). p29b, with its elastinolytic activity, may contribute to tissue injury, as has been proposed for proteinase 3 (5, 20). Antimicrobial proteins with restricted or minimal proteolytic activity, like azurocidin, could theoretically supplement host defense against infection without increasing the content of potentially destructive proteases in the lysosomal granules. The relationship between azurocidin and p29b is analogous to that between eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN), two 18-kD proteins of the eosinophil granule. By amino acid sequencing both are members of the RNase family (46). ECP is a potent helminthotoxin with little RNase activity, while EDN has considerable RNase and little antimicrobial activity (47). Comparison of the structures of azurocidin and p29b may provide insights into the features required for killing of particular pathogens and those contributing to protease function and its specificity.

Note added in proof. After submission of this manuscript for publication, it was reported that Wegener's granulomatosis autoantibodies identify a 29-kD, DFP-binding neutrophil protein whose amino terminal sequence is identical with p29b in 18 of 20 residues (48, 49). The amino terminal sequence of azurocidin appears to be identical with all 20 amino terminal residues of CAP 37 (50) and 15 of 16 residues of an ~ 30-kD protein isolated from human kidney stones (51).

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