INFECTION AND IMMUNITY, Sept. 1992, p. 3556–3565
0019-9567/92/093556-10$02.00/0

Vol. 60, No. 9

Cryptdins: Antimicrobial Defensins of the Murine Small Intestine

PATRICIA B. EISENHAUER,[1] SYLVIA S. S. L. HARWIG,[1] AND ROBERT I. LEHRER[1,2]*

Department of Medicine, UCLA School of Medicine, Los Angeles, California 90024,[1] and Department of Medicine, Wadsworth-UCLA Veterans Administration Hospital, Los Angeles, California 90025[2]*

Received 6 April 1992/Accepted 4 June 1992

Paneth cells are specialized small intestine epithelial cells that contain lysozyme, possess phagocytic properties, and secrete cytoplasmic granules into the intestinal crypt lumen after the entry of bacteria. Recent studies by Ouellette and associates (A. J. Ouellette, R. M. Greco, M. James, D. Frederick, J. Naftilan, and J. T. Fallon, J. Cell Biol. 108:1687–1695, 1989) indicated that murine Paneth cells produce prodefensin mRNA, but the properties of its peptide product were not reported. We purified two closely related defensins, cryptdin 1 and cryptdin 2, from a subcellular fraction of murine small intestine cells that was enriched in Paneth cells. Both peptides contained 35 amino acid residues, including the characteristic defensin "signature" of six invariantly conserved cysteines. Cryptdins 1 and 2 were approximately 90 to 95% homologous to each other and to the carboxy-terminal domain of the 93-amino-acid defensin precursor, cryptdin A, described by Ouellette and associates (Ouellette et al., J. Cell Biol. 108:1687–1695, 1989). Both cryptdins exerted bactericidal activity against *Listeria monocytogenes* EGD and *Escherichia coli* ML-35p in vitro. Their potency exceeded that of human neutrophil defensin HNP-1 but was considerably lower than that of NP-1, a defensin produced by rabbit neutrophils and alveolar macrophages. Both cryptdins killed mouse-avirulent *Salmonella typhimurium* 7953S (*phoP*) much more effectively than its *phoP*$^+$, mouse-virulent, isogenic counterpart, *S. typhimurium* 14028S. Our data indicate that mouse intestinal prodefensins are processed into 35-amino-acid mature defensins (cryptdins) with broad-spectrum antimicrobial properties. The production of defensins and lysozyme by Paneth cells may enable them to protect the small intestine from bacterial overgrowth by autochthonous flora and from invasion by potential pathogens that cause infection via the peroral route, such as *L. monocytogenes* and *Salmonella* species.

Antimicrobial polypeptides are widely distributed within animal tissues and cells that frequently encounter microorganisms. Among these endogenous antibiotic substances are defensins (27), cecropins (2, 25), magainins (58), and tracheal antimicrobial peptide (TAP) (7), a peptide produced by bovine tracheal epithelial cells. Although defensins were originally discovered in mammalian neutrophils and macrophages (reviewed in reference 16), certain specialized epithelial cells of the murine small intestine, known as Paneth cells (PC), recently were shown to contain mRNA that encoded a 93-amino-acid peptide (cryptdin A) whose carboxy-terminal end contained a typical defensin motif (37). Myeloid defensins are formed from preprodefensins by sequential intracellular proteolysis that, when completed, generates mature defensins with 29 to 34 amino acid residues, including 0 to 2 amino acid residues that are amino-terminal to their initial cysteine (16, 21, 54). Although many myeloid defensins manifest potent broad-spectrum antimicrobial activity, some exert other biological effects, including cytotoxicity (29, 50), competitive inhibition of adrenocorticotropin-mediated sterol production by adrenal cells (59, 60), inhibition of protein kinases (4), and selective chemotaxis for monocytes (52). Neither the structure(s) of the mature (i.e., processed) murine small intestine defensins nor their antimicrobial activities have been reported.

PC constitute 3 to 7% of the epithelial cells in small intestine crypts (5) and are restricted to their basal portion. Although PC are found in most mammals, they are curiously absent from cats and related carnivores (56). PC contain an elaborate endoplasmic reticulum, a highly developed Golgi region, and many large (1- to 3-μm) apical secretory granules (53) that contain lysozyme (6). They also contain immunoglobulin A (43), phospholipase A2 (49), dipeptidyl peptidase (40), epidermal growth factor (38), and tumor necrosis factor mRNA (23), equipping them to exert wide-ranging effects on the small intestine function. The lysozyme found in mouse PC granules is a distinct isoform (P-lysozyme) whose primary structure differs from that of the less cationic M-lysozyme that is present in macrophages, granulocytes, and many secretions (14, 22). Since murine P-lysozyme does not have a counterpart in rats, it may have evolved relatively recently (19).

Although the functions of PC are uncertain, circumstantial evidence supports their participation in small intestine antibacterial defenses. In many species, PC granules possess a core that is rich in basic proteins and surrounded by a peripheral mantle that is composed of polysaccharides (45, 51). PC can ingest certain microorganisms (11) and protozoans (12), and they secrete cytoplasmic granules into the intestinal crypt lumen, especially after live or heat-killed bacteria enter the gut (42, 44). As degranulation by murine PC is inhibited by atropine and stimulated by pilocarpine, it appears to be under cholinergic control (1, 41). Their secretory and phagocytic abilities, combined with their strategic location in small intestine crypts, could allow PC to defend against bacterial overgrowth or invasion in this exclusive site of epithelial cell renewal. To further assess the possibility that PC participate in the antimicrobial defenses of the small intestine, we have isolated defensins from the murine small intestine, ascertained their primary structures, and tested their antibacterial properties. This report describes our findings.

* Corresponding author.

MATERIALS AND METHODS

Intestinal cell preparation. Female adult Swiss Webster mice, obtained from Harlan Sprague-Dawley Inc., Indianapolis, Ind., were subjected to fasting but allowed to drink water ad libitum for 12 h before being sacrificed. Cells were isolated from the small intestine by two methods. The first of these, used in our initial studies, incorporated elements of two previously described procedures (24, 55). In brief, the entire small intestine was removed and cut into four equal-length segments that were flushed with 0.9% NaCl containing 1 mM dithiothreitol to remove luminal contents. Each segment was carefully everted onto a 0.1-ml glass pipette, which was then placed in a tube containing phosphate-buffered saline (PBS) (pH 7.4) with 27 mM sodium citrate. After 15, 30, and 45 min, the solution was removed and replaced with PBS that contained 0.5 mM dithiothreitol and 1.5 mM EDTA. The exfoliated cell preparation obtained at between 45 and 60 min and expected to be relatively enriched in PC was washed twice with PBS with 1 mM EDTA. The cells were resuspended in ice-cold 5% acetic acid, subjected to three 15-s cycles of sonication, and then stirred overnight at 4°C. Thereafter, the material was centrifuged at 27,000 × *g* for 20 min at 4°C, and the supernatant was placed in Spectra/por 3 tubing (Spectrum Medical Industries, Los Angeles, Calif.) and dialyzed against 5% acetic acid.

This procedure was later simplified by replacing the four 15-min incubations with two periods of 30 min each. Both cell-rich solutions were combined and centrifuged at 450 × *g* for 10 min at 4°C to separate the supernatant (fraction 1) from the exfoliated intestinal cells (fraction 2). The latter were suspended in 5 ml of ice-cold 0.34 M sucrose (pH 7.4), and the suspension was disrupted in a Potter-Elvejhem homogenizer until >90% cellular breakage was achieved, as judged by phase-contrast microscopy. The homogenate was centrifuged at 450 × *g* for 10 min at 4°C, and the resulting pellet was washed twice in 5 to 10 ml of 0.34 M sucrose. The 450 × *g* sucrose supernatant was centrifuged at 27,000 × *g* for 20 min to separate the supernatant (fraction 2s) from the granule-rich pellet (fraction 2p). The remaining small intestine tissue was processed by a modification of the method of Hammer et al. (19, 20). In brief, the remaining small intestine was weighed, minced with a razor blade into 1- to 2-mm pieces, and homogenized in cold 2% acetic acid (2 ml/g) with a Potter-Elvejhem homogenizer. The homogenate was centrifuged at 13,000 × *g* for 15 min, and the clear supernatant (fraction 3) was reserved. Fraction 2p obtained from 84 mice was used for our isolation and characterization of cryptdins.

Peptide purification. The granule-rich sediment was suspended in ice-cold 5% acetic acid and sonicated three times, for 12 s each time, with a Bronwill Biosonik IV sonicator (VWR Scientific, Los Angeles, Calif.). This sonicate was stirred for 3 h at 4°C and then centrifuged at 27,000 × *g* for 15 min. The insoluble residue was reextracted by being stirred overnight at 4°C with 5% acetic acid. After centrifugation at 27,000 × *g* to obtain the second extract, both supernatants were combined, concentrated by vacuum centrifugation, and stored at −20°C. Thawed concentrates were chromatographed on a SynChropak GPC 100 gel permeation column (Synchrom, Inc., Lafayette, Ind.) and eluted at a flow rate of 0.5 ml/min with 100 mM ammonium acetate–1% acetic acid in 10% acetonitrile (pH 4.99). Fractions (1 ml) were collected, and aliquots were tested for microbicidal activity against *Listeria monocytogenes* EGD and by polyacrylamide gel electrophoresis (PAGE) on acid-urea (AU)- and sodium dodecyl sulfate (SDS)-polyacrylamide gels. Fractions of interest were pooled, concentrated by vacuum centrifugation, and further purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on a Vydac C-18 column (4.6 mm by 25 cm; The Separations Group, Hesperia, Calif.) with water-acetonitrile gradients that contained 0.1% trifluoroacetic acid. Final RP-HPLC purifications were accomplished by use of more shallow water-acetonitrile gradients with 0.13% heptafluorobutyric acid (HFBA) as the ion-pairing agent.

Amino acid analysis. Performic acid-oxidized or native peptides were hydrolyzed with 6 N hydrochloric acid in an evacuated, sealed tube at 110°C for 40 h. Amino acids were quantitated as phenylthiocarbamyl derivatives by RP-HPLC on a C18 column as previously described.

Sequence determination. S carboxamidomethylation of dithiothreitol-reduced peptides was accomplished with iodoacetamide in a solution containing 6.0 M guanidine hydrochloride, 0.5 M Tris hydrochloride (pH 8.1), and 0.2 mM EDTA, and the peptides were desalted on a small-bore Vydac C-18 column (2.1 mm by 15 cm; The Separations Group). For determination of the primary amino acid sequence, approximately 0.3 nmol of the native or S-carboxamidomethylated peptides was sequenced on a model 475A sequencing system (Applied Biosystems, Inc., Foster City, Calif.) by standard procedures. In some early experiments, peptides that had been excised from AU-polyacrylamide gels were eluted, subjected to SDS-PAGE, electroblotted onto polyvinylidene difluoride membranes, and sequenced directly (31).

Antimicrobial testing. *Escherichia coli* ML-35p (26) and *S. typhimurium* 14028S and 7953S (gifts from Fred Heffron, University of Oregon) were maintained on Trypticase soy agar plates (containing 15 μg of tetracycline per ml for *S. typhimurium* 7953S). *L. monocytogenes* EGD (generously provided by Pieter Hiemstra, University of Leiden) was kept on Trypticase soy agar with 5% sheep erythrocytes (Clinical Standards Lab Inc., Rancho Dominguez, Calif.). Bacterial cultures were grown overnight in Trypticase soy broth at 37°C, diluted 1:1,000 (1:100 for *L. monocytogenes*) into fresh Trypticase soy broth, and incubated for 2.5 h at 37°C with shaking. The number of CFU was estimated by spectrophotometry (A_{620}), referenced to previously determined standards.

Gel overlay assay. Samples, typically crude or partially purified extracts, were subjected to AU-PAGE as previously described (28). In brief, the gels were rinsed for 15 min in 10 mM sodium phosphate buffer (pH 7.4) and placed on top of a 1-mm-deep agar layer that had been seeded with approximately 4 × 10^5 bacteria per ml. In addition to bacteria, this agar underlayer contained 0.03% (wt/vol) trypticase soy broth powder, 1% (wt/vol) agarose, 10 mM sodium citrate-phosphate buffer, and 0.02% Tween 20. Stock citrate-phosphate buffer was made by mixing 100 mM citric acid and 100 mM sodium phosphate and diluting this mixture 1:10 into the agar underlayer. The final pH of the agar underlayer was adjusted to 6.5 or 7.4 before the agar was autoclaved, and the bacteria were added after the agar had cooled to 42 to 43°C, just before being poured. After 3 h of incubation at 37°C to allow the electrophoresed proteins to diffuse into the bacterial underlayer, the AU-polyacrylamide gel was removed and a nutrient-rich top agar was poured over the agar underlayer to allow the surviving bacteria to grow. After incubation for at least 24 h at 37°C, the presence of a clear zone indicated the locations of antimicrobial polypeptides.

Radial diffusion assay. Sixteen equally spaced 3-mm wells were prepared in the agarose underlayer, which was other-

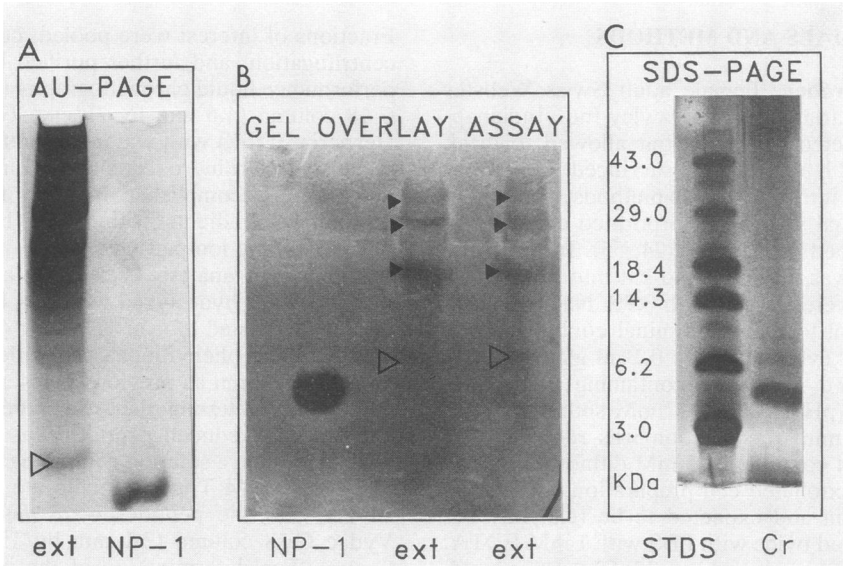

FIG. 1. Identification of cryptdin. (A) AU-polyacrylamide gel stained with Coomassie brilliant blue R-250. The left lane contained 42.5 μg of protein, obtained by extracting the 45- to 60-min fraction of exfoliated murine small intestine cells. The open triangle shows the band subsequently identified as cryptdin. The right lane contained 1 μg of rabbit defensin NP-1. (B) The dark bands identify components with bactericidal activity against *L. monocytogenes* EGD in the original AU-polyacrylamide gel (data not shown). The left lane contained 1 μg of NP-1, which caused the large dark (bacterium-free) zone. The other two lanes contained approximately 42.5 μg of the same small intestine cell extract as that in panel A. Note that several components with bactericidal activity were present (solid triangles), in addition to cryptdin (open triangles). (C) Silver stain. The band identified as cryptdin in the right AU-polyacrylamide gel was excised, solubilized, and run on this SDS-polyacrylamide gel. The left lane contained protein standards whose masses are shown in kilodaltons. The right lane contained the cryptdin band, whose estimated mass was 5.1 kDa.

wise prepared as described above. Lyophilized samples were dissolved in 0.01% acetic acid and added in 5-μl portions to the wells. The plates were incubated for 3 h at 37°C to allow the peptides to diffuse radially into the agarose underlayer. A nutrient-rich overlay was poured, and the plates were incubated for at least 18 h at 37°C to permit the surviving bacteria to form colonies. The appearance of a zone of clearance around a well denoted bactericidal activity. Its diameter was measured and recorded in units (10 units = 1 mm). Because only a few micrograms of purified cryptdins were available for antimicrobial testing, we used the ultrasensitive radial diffusion assay to compare their antibacterial activities with those of human HNP-1 and rabbit NP-1, which we had purified to homogeneity from human peripheral blood neutrophils and rabbit peritoneal exudate neutrophils, respectively.

Other methods. Protein was measured by the bicinchoninic acid procedure (Pierce Chemicals, Rockford, Ill.) with bovine serum albumin as a standard. AU-PAGE was performed by use of a minigel format with a model SE 250 vertical gel unit (Hoefer, San Francisco, Calif.). SDS-PAGE was performed by use of a 16.5% acrylamide–tricine–SDS system (48) with the same apparatus. Gels were stained with a solution containing 1 g of Coomassie brilliant blue R-250 (Sigma), 270 ml of methanol, 630 ml of water, and 150 ml of formaldehyde and destained with a methanol-water-formaldehyde solution (1:3:0.04). Silver staining of the SDS-polyacrylamide gels was performed by the method of Wray et al. (57). P-lysozyme and M-lysozyme, which migrated identically on AU-polyacrylamide gels, were resolved on Reisfield-Williams gels, which were prepared essentially as described by Hammer and colleagues (19). In this system, the relative cathodal migrations of P-lysozyme, M-lysozyme, and hen egg white lysozyme were 0.60, 0.42, and 0.52, respectively. These cathodal migration values were calculated relative to that of an NP-1 standard as follows: millimeters of migration of lysozyme/millimeters of migration of NP-1.

Amino acid sequence accession number. The amino acid sequences of cryptdins 1 and 2 have been submitted to PIR (National Biomedical Research Foundation, Washington, D.C.) and have been assigned accession numbers A 37305 and B 37305, respectively.

RESULTS

Initial studies. If the previously described 93-amino-acid intestinal preprocryptdin (37) were processed like mature myeloid defensins (54), mature cryptdin should contain 30 to 32 amino acid residues and possess a mass of approximately 4.0 kDa. Its polar residues (six arginines [R], two lysines [K], one histidine [H], one glutamic acid [E], and zero to one aspartic acid [D]) should give it a net charge ([R + K + H] - [E + D]) of +7 to +8 under the acidic conditions used in AU-PAGE. Consequently, its migration in AU-PAGE should be less cathodal than that of rabbit defensin NP-1 (33 amino acids; net charge, +10) and closely resemble that of rabbit defensin NP-3a (34 amino acids; net charge, +8) or rat defensin RatNP-2 (32 amino acids; net charge, +7) (16).

In our initial attempts to identify such a peptide, we used a labor-intensive sequential exfoliation method to prepare a cell fraction that was relatively enriched in PC. Acetic acid extracts of these cells contained a prominent band whose migration relative to that of NP-1 in AU-PAGE was as predicted for mature cryptdin (Fig. 1A), although the apparent mass in SDS-PAGE (approximately 5.1 kDa) was somewhat higher than expected (Fig. 1C). This peptide showed bactericidal activity against *L. monocytogenes* EGD in our gel overlay assays, although we could not fail to notice that several other, less cationic bands manifested even greater

TABLE 1. Recovery of small intestine protein and lysozyme in combined materials obtained from 20 mice[a]

Fraction	Protein, mg (% of total)	Lysozyme, μg (% of total)	Ratio (μg/mg)
1	504.6 (42.7)	3,034.5 (63.1)	6.01
2p	83.3 (7.0)	349.0 (7.3)	4.19
2s	480.0 (40.6)	444.0 (9.2)	0.93
3	113.4 (9.6)	981.2 (20.4)	7.94
Total	1,181.3 (99.9)	4,808.7 (100.0)	4.07

[a] Protein content was measured by the bicinchoninic acid assay. Lysozyme was measured by radial diffusion in gels with hen egg white lysozyme standards. Its quantity is expressed in terms of hen egg white lysozyme equivalents.

activity (Fig. 1B). We excised the putative cryptdin band from AU-polyacrylamide gels and subjected it to SDS-PAGE, polyvinylidene difluoride membrane transfer, and gas-phase microsequencing. The N-terminal sequence of the peptide (LRDLV-Y-RARG-K-RE--N) was homologous to residues 59 to 78 of mouse (*Mus musculus*) preprocryptdin A (LRDLVCYCRSRGCKGRERMN), except that the Ser-68 in preprocryptdin A was replaced by an alanine. Overall, we were satisfied that we had identified a mature form of cryptdin or a cryptdin homolog, and we set out to prepare it in larger amounts. Our studies of the other antimicrobial components present in Fig. 1B will be the subject of a future report.

As described in Materials and Methods, we simplified the cell preparation method to generate fewer fractions and selected a 27,000 × *g* granule fraction from a sucrose cell homogenate (fraction 2p) for our purification of cryptdin. As shown in Table 1, fraction 2p contained approximately 7.3% of the total lysozyme recovered from the small intestine. Over 90% of its lysozyme was the fast-migrating, PC-specific P isoform, confirming that PC granule components were present in this fraction. The specific activity of lysozyme in fraction 2p (4.19 μg/mg of protein) compared favorably with its specific activity in PC granules (1.74 μg/mg) that had been prepared from sucrose cell homogenates of NMRI mouse small intestines by an alternative, but related, procedure (6). Even the simplified gut fractionation procedure was very labor-intensive, and fraction 2p yielded relatively small amounts of protein, some of which was undoubtedly of non-PC origin. Although our overall recovery of cryptdin was only 1 to 2 μg per mouse, in the future it should be possible to enhance cryptdin recovery by using fractions 1, 2s, and 3 as supplemental starting materials.

Peptide purification. We purified the candidate cryptdin molecule by RP-HPLC, beginning with gel permeation with a SynChropak GPC 100 column (Fig. 2). Column fractions were tested for antimicrobial activity against *L. monocytogenes* EGD by the radial diffusion assay (data not shown) and were analyzed by AU-PAGE. In AU-PAGE, stained bands with appropriate cationic properties were most prominent in fractions 20 to 23 (Fig. 2). To minimize any loss of cryptdin, we pooled fractions 18 to 25 and further fractionated them by RP-HPLC. We recovered the cryptdin candidates only in fractions 28 and 29 (Fig. 3), which again showed microbicidal activity against *L. monocytogenes* EGD in the radial diffusion assay (data not shown). AU-PAGE analysis of fraction 28 revealed two narrowly separated components. The faster (more cathodal) component was designated cryptdin 1, and the slower component was designated cryptdin 2. Only one species, whose migration in AU-PAGE corresponded to that of cryptdin 2, was present in fraction 29. Fractions 28 and 29 were purified by additional cycles of RP-HPLC with shallower gradients and an alternative ion-pairing agent (0.13% HFBA) with a water-

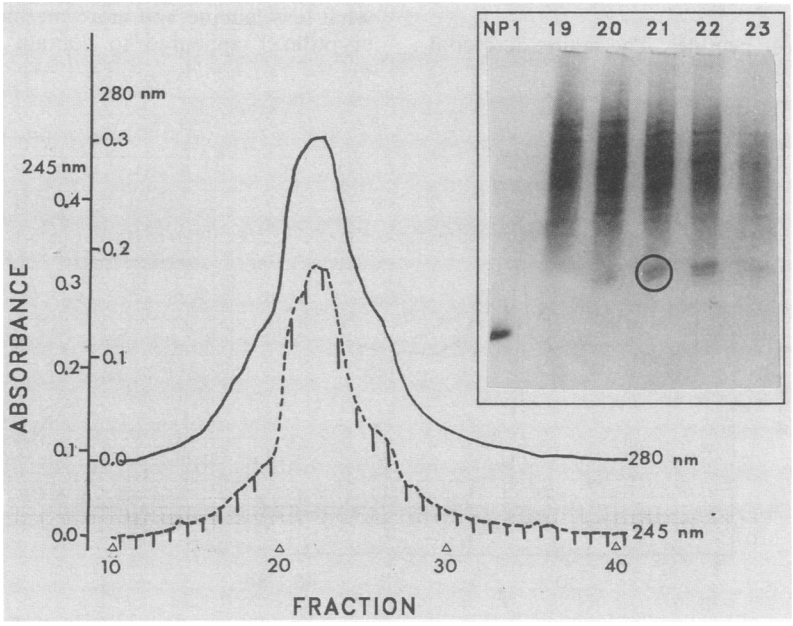

FIG. 2. Gel permeation chromatography. Fraction 2p was prepared from 20 mice and concentrated to 5.0 ml. Approximately 0.65 ml was applied to a Synchropak GPC 100 column and eluted at 0.5 ml/min with monitoring as shown. The effluent was monitored at 245 and 280 nm, and fractions (delineated by tick marks on the 245-nm tracing) were collected every 2 min. Aliquots of each fraction were examined by AU-PAGE (inset). Cryptdin was most prominent in fraction 21 (circled) but was also evident in fractions 20, 22, and 23. Fractions 18 to 25 were pooled for further purification by RP-HPLC.

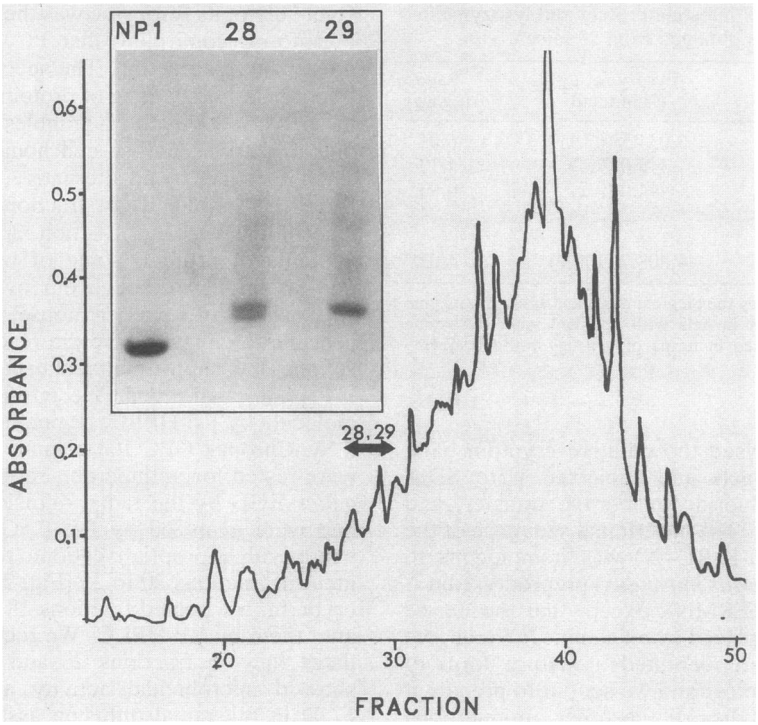

FIG. 3. RP-HPLC. Pooled fractions 18 to 25 (Fig. 2) were concentrated and chromatographed on a C-18 column with a linear gradient of 0 to 60% acetonitrile in 0.1% trifluoroacetic acid over 60 min. Fractions were collected each minute, and aliquots were analyzed by AU-PAGE (inset). The cryptdin candidates were found in fractions 28 and 29.

acetonitrile gradient. Under these conditions, cryptdin 1 was partially resolved into the two semidistinct peaks shown in Fig. 4; we did not further separate these peaks. Cryptdin 2 appeared more homogeneous under these conditions. The final RP-HPLC purification of cryptdin 2 is shown in Fig. 5.

Characterization of mouse peptides. The amino acid compositions of cryptdins 1 and 2 are shown in Table 2. Overall, their relative amino acid contents agreed with the reported cDNA sequence data for murine cryptdins. Both cryptdins were rich in cysteine (which is underestimated by acid hydrolysis) and arginine. However, both contained somewhat less leucine and more methionine than expected, and cryptdin 1 appeared to contain one more arginine than

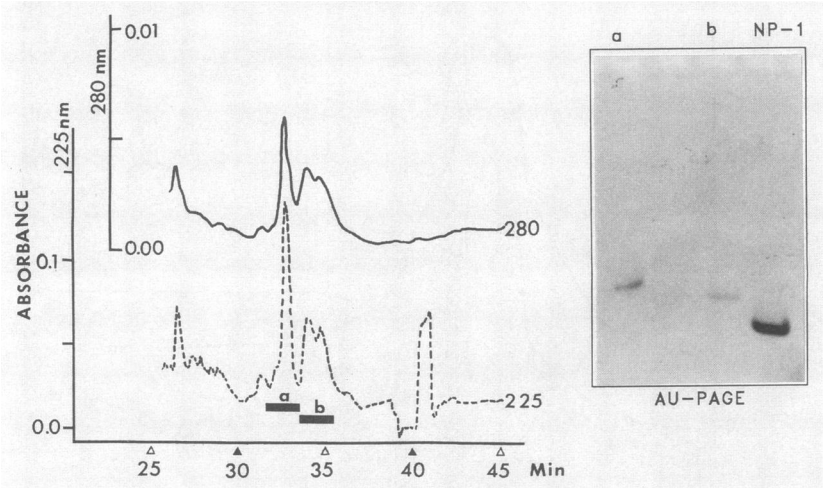

FIG. 4. Further purification of fraction 28. Fraction 28 (Fig. 3), derived from approximately 12 mice, was dissolved in 135 μl of 0.01% acetic acid, applied to a small-bore C-18 column, and eluted with the following gradient of acetonitrile (ACN) in 0.13% HFBA: 15% ACN, from 0 to 1 min; 15 to 35% ACN, from 1 to 21 min; 35 to 50% ACN, from 21 to 51 min; 50 to 75% ACN, from 51 to 56 min. The peptides of interest were eluted in two peaks, indicated by the bars labelled a and b on the chromatogram. Peak a (cryptdin 2) emerged at 32 to 33 min, and peak b (cryptdin 1), which was bifid, emerged at 34 to 35 min. The inset shows AU-PAGE analysis of these peaks, containing approximately 1 μg of NP-1 and 0.3 to 0.5 μg of cryptdins 1 and 2. The gel was stained with Coomassie brilliant blue R-250.

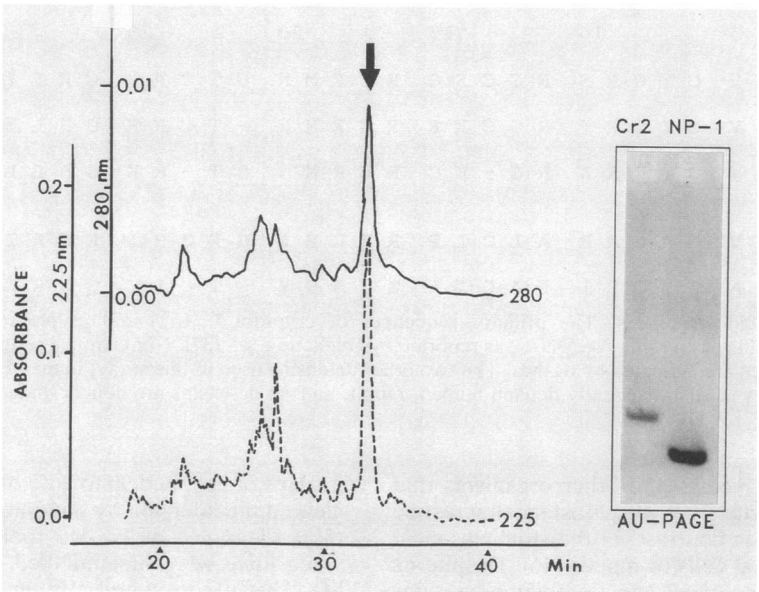

FIG. 5. Final purification of fraction 29. Fraction 29 (Fig. 3) from approximately 12 mice was dissolved in 135 μl of 0.01% acetic acid, applied to a small-bore C-18 column, and eluted with the acetonitrile-HFBA gradient described in the legend to Fig. 4. The peptide of interest (cryptdin 2) was eluted as a single peak at 32 to 33 min (arrow). The inset, an AU-polyacrylamide gel stained with Coomassie brilliant blue R-250, contained approximately 1 μg of NP-1 and 0.5 μg of cryptdin 2.

cryptdin 2. The amino acid sequences of cryptdins 1 and 2 are shown in Fig. 6.

Because of our miniscule supplies of the purified cryptdins and some technical misadventures, several residues in each peptide still remain to be definitively identified. Nevertheless, it can be seen that cryptdin 1 differed from the peptide encoded by the previously reported cDNA sequence in that it contained threonine instead of serine at residue 10, arginine instead of glycine at residue 15, and methionine instead of leucine at residue 29. Cryptdin 2 was homologous to cryptdin 1 but differed in residues 10 (alanine) and 31 (methionine). Unlike cryptdin 1, but like the reported cDNA sequence-encoded peptide, it contained glycine at residue 15 and leucine at residue 29. During these purifications, we also obtained small amounts of a third intestinal defensin whose migration in AU-PAGE was more cathodal than that of cryptdin 1. Partial N-terminal amino acid analysis of this peptide revealed that residues 1 to 9 were identical to those of the other cryptdins shown in Fig. 6 and that residue 10 was an arginine. These data indicate that the products of the mouse cryptdin gene(s) show considerable polymorphism and suggest that residue 10 may be a "hot-spot" for genetic change.

TABLE 2. Amino acid analysis of cryptdins

Amino acid	Cryptdin 1		Cryptdin 2		No. of residues in the cDNA sequence
	Mol%	Residues/molecule[a]	Mol%	Residues/molecule[a]	
Alanine	1.74	0.60	3.39	1.18	0
Arginine	19.72	6.90	17.21	6.03	7
Asp(x)[b]	6.69	2.34	7.02	2.46	2
Cysteine[c]	13.98	4.90	12.47	4.36	6
Glu(x)[d]	4.76	1.67	5.20	1.83	1
Glycine	7.93	2.77	10.36	3.63	4
Histidine	6.18	2.16	4.40	1.54	1
Isoleucine	0.15	0.05	0.30	0.11	0
Leucine	7.42	2.59	11.50	4.00	5
Lysine	6.44	2.25	6.63	2.32	2
Methionine	5.81	2.03	4.56	1.60	1
Phenylalanine	0.06	0.05	0.33	0.12	0
Proline	1.93	0.67	1.99	0.70	0
Serine	1.92	0.67	2.57	0.90	1
Threonine	7.67	2.69	4.34	1.52	2
Tryptophan	ND[e]	ND	ND	ND	0
Tyrosine	5.14	1.80	5.12	1.79	2
Valine	2.37	0.82	2.61	0.92	1
Total	99.91	34.97	100.00	35.01	35

[a] Calculated on the assumption that the mature cryptdins contained 35 amino acids.
[b] Asp(x), aspartic acid and asparagine.
[c] Measured as cysteic acid in a separate, performic acid-oxidized sample.
[d] Glu(x), glutamic acid and glutamine.
[e] ND, not determined.

Antimicrobial activity. We used an ultrasensitive radial diffusion assay to assess the antimicrobial properties of cryptdins 1 and 2. Figure 7 illustrates such an assay. Since only 5-μl defensin samples were added to each well, the complete standard curves (5 to 500 μg/ml) for the HNP-1 and NP-1 controls required only 5 μg of each of these peptides. These plates also contained a 100-μg/ml sample of cryptdin 2, which was added to well D2. Figure 8 compares the activities of cryptdins 1 and 2, rabbit NP-1, and human HNP-1 against *L. monocytogenes* and *E. coli*. Note that although both mouse intestinal defensins were more active than human defensin HNP-1, they were less active than rabbit defensin NP-1. Figure 9 shows that neither cryptdin killed mouse-virulent, *phoP*$^+$ *S. typhimurium* 14028S, although both killed its avirulent, isogenic, *phoP* congener, *S. typhimurium* 7953S.

DISCUSSION

The autochthonous small intestine flora of mice and humans consists mostly of lactobacilli and anaerobic strepto-

```
      1        5         10        15        20        25        30        35
cDNA  L R D L V  C Y C R S  R G C K G  R E R M N  G T C R K  G H L L Y  T L C C R
Cr1   L R D L V  C Y C R T  R G C K R  R E R M N  - T C R K  G H L M Y  T L C C -
Cr2   L R D L V  C Y C R A  R G - K G  R E R M N  G T - R K  G H L L Y  M - - - -

NP1         V V  C A C R R  A L C L P  R E R R A  G F C R I  R G R I H  P L C C R R
HNP1          A  C Y C R I  P A C I A  G E R R Y  G T C I Y  Q G R L W  A F C C
```

FIG. 6. Primary amino acid sequences. The primary sequences of cryptdin 1 (Cr1) and cryptdin 2 (Cr2) are aligned with the carboxy-terminal 35 amino acids of cryptdin A (cDNA), as reported by Ouellette et al. (37). Nonidentical residues are shown in boldface type, while still unidentified residues are indicated by dashes. The two other defensins used in this study, human HNP-1 and rabbit NP-1, are also shown. The invariant residues in all 14 currently defined human, rabbit, and rat defensins are doubly underlined in HNP-1.

cocci, leavened by a few yeasts and other organisms that originate from the oral cavity (8, 9, 47). Most small intestine bacteria are intraluminal; in contrast, in the stomach, most bacteria adhere to epithelial cells or mucus (46). Despite its nutrient-laden contents, the small intestine lumen contains $<10^4$ bacterial CFU/ml (17). The factors responsible for these low bacterial populations are not well understood but are traditionally stated to include peristalsis, bile salts, and the sterilizing actions of gastric acid. The possibility that endogenous antimicrobial peptides or proteins other than lysozyme might help to maintain these low population densities has received relatively little attention.

Although it is very difficult to establish enteric pathogens such as *Vibrio cholera*, *Shigella* spp., *Salmonella* spp., and staphylococci in the gut of a healthy adult mouse (47), it is considerably less difficult to infect neonatal mice (36). Mushin and Dubos used an enteropathogenic human strain of *E. coli* to show age-dependent intestinal resistance in specific-pathogen-free Swiss mice (36). Although young mice (up to 13 days old) were uniformly colonized, older mice that were infected under the same conditions became progressively more difficult to colonize. Intestinal carriage of *E. coli* in mice colonized soon after birth declined sharply by 21 days of age, and 90 to 96% of the animals had completely cleared the infection by the time of weening, at 24 to 28 days (36). Clearance of *E. coli* took place almost immediately when mice were first infected during their fourth week of life. The authors stated, "It appears that some change which occurred in the animal around weaning time brought about a sudden increase in its resistance to *E. coli*."

Similar observations were recorded almost a century ago, when Metchnikoff (33) wrote, "Many animals may, without running the least risk, swallow large numbers of bacteria which in man produce grave gastrointestinal disease. . . . We may recall the difficulties which so many investigators have met with in inducing intestinal cholera in laboratory animals, which are so refractory to Koch's vibrio. Only very young animals, especially unweaned rabbits, are capable of contracting fatal intestinal cholera. . . . As soon as rabbits begin to feed on vegetables, they acquire an immunity which is insuperable."

The ontogeny of PC parallels the acquisition of small intestine resistance. In rats, lysozyme-containing PC were found in <1% of jejeunal and ileal crypts at 3 to 6 days of age, in 30 to 45% of crypts at 2 weeks of age and in 77 to 93% of crypts by the end of the third week (30). In mice,

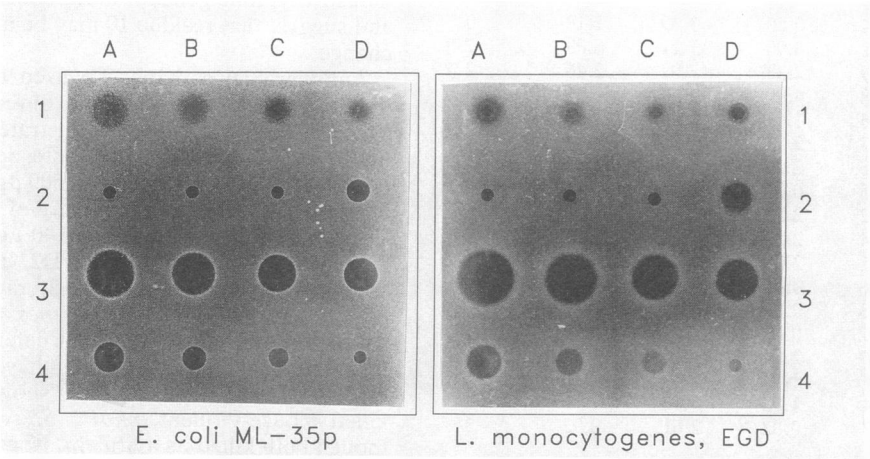

FIG. 7. Radial diffusion assays. *E. coli* ML-35p (left panel) and *L. monocytogenes* EGD (right panel) were incorporated into 1-mm-thick agarose gels that contained 16 evenly spaced wells, 3 mm in diameter. Bactericidal activity was tested by placing 5-μl defensin samples (in 0.01% acetic acid) in each well, incubating them for 3 h to permit the defensins to diffuse into the agarose, and pouring a thin nutrient-rich overlay that allowed the surviving bacteria to grow and form colonies. The placement and concentration (in micrograms per milliliter) of HNP-1 samples were as follows: A1, 500; B1, 250; C1, 100; D1, 50; A2, 25; B2, 10; and C2, 5. The placement and concentration (in micrograms per milliliter) of NP-1 samples were as follows: A3, 500; B3, 250; C3, 100; D3, 50; A4, 25; B4, 10; and C4, 5. Well D4 received 5 μl of 0.01% acetic acid, and well D2 received 5 μl of cryptdin 2 (100 μg/ml).

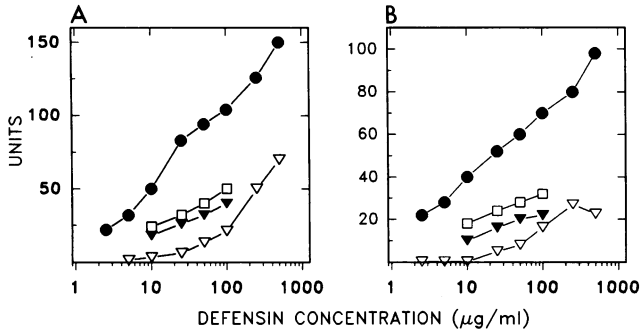

FIG. 8. Activities of cryptdins against *L. monocytogenes* EGD (A) and *E. coli* ML-35p (B). Radial diffusion assays were performed at pH 7.4, and the zone diameters were measured and expressed in units (10 units = 1 mm). Symbols: ▼, cryptdin 1; □, cryptdin 2; ●, NP-1; ▽, HNP-1. Peptide concentrations were measured by the bicinchoninic acid assay. The control, 0.01% acetic acid, gave no zone of clearance.

lysozyme-containing PC first appeared in intestinal crypts by approximately 7 days of life (32). Similar results were obtained when cryptdin mRNA was used as a PC marker (37). Whereas cryptdin mRNA was present in only 5 to 10% of crypts of 10-day-old mice, higher levels were observed in 70 to 80% of crypts in 16-day-old mice and adult levels were present in 100% of crypts in 20-day-old mice (37).

The two closely related forms of cryptdin described in this report were obtained from a preparation of murine small intestine cells that contained PC granules. Both contained 35-amino-acid residues and differed only slightly from each other and from the carboxy-terminal 35 amino acids of cryptdin A mRNA (37). Because cryptdins 1 and 2 were purified from pooled material derived from the small intestines of over 80 outbred mice, they may be products of allelic genes or indications that the murine cryptdin gene locus has undergone reduplication. Both conditions have precedents with respect to myeloid defensins; the former accounts for the polymorphic expression of defensins RatNP-1 and RatNP-2 in outbred rats (10), and the latter accounts for the production of both MCP (NP)-1 and MCP (NP)-2 in outbred New Zealand White rabbits (15).

All previously characterized mature myeloid defensins have contained no more than 34 amino acids, including 0 to 2 amino acid residues preceding (amino-terminal to) the first cysteine (16). In contrast, cryptdins 1 and 2 contained 35 amino acids, including 5 amino acids preceding the first cysteine. Although the processing steps involved in deriving cryptdins 1 and 2 from their prodefensin precursors (37) remain to be defined, the process clearly differs somewhat from that recently described for human neutrophil defensins (54). The possibility that additional forms of these intestinal defensins, such as the secreted 75-amino-acid prodefensins of human neutrophils (54; unpublished data), are produced deserves study, as does the possibility that further amino-terminal processing of cryptdins 1 and 2 might occur by intestinal or pancreatic proteases in the intestinal lumen.

We found that cryptdins 1 and 2 killed our test strains of *E. coli*, *S. typhimurium*, and *L. monocytogenes* more effectively than human neutrophil defensin HNP-1. Of particular interest was the observation that the cryptdins killed *S. typhimurium* 7953S (*phoP*), which is avirulent for mice via parenteral and oral routes, whereas they were inactive against its isogenic, mouse-virulent counterpart, *S. typhimurium* 14028S (*phoP*$^+$). These results support earlier demonstrations that the sensitivity of *S. typhimurium* to defensins and its virulence for mice are linked and that both properties are controlled by the two-component *phoP-phoQ* regulon (13, 18, 35), which controls the expression of several genes, including *pagC*, whose product is an 18-kDa cationic protein with homology to the *ail* gene product of *Yersinia enterocolitica* (39). Although *S. typhimurium* with mutations in *pagC* was reported to be avirulent for mice and deficient in survival within murine macrophages, its resistance to rabbit defensin NP-1 was intact (34). Given the host specificity of salmonelloses (*S. typhimurium* is used as a surrogate for *S. typhi* in experimental models of typhoid because the latter organism is avirulent for mice [3]), comparable studies with *pagC* mutants and murine defensins would also be of interest.

The present demonstration that the mouse small intestine produces antimicrobial defensins further strengthens the evidence that PC play a role in intestinal tract host defense. Although the presence of small intestine defensins in humans and other mammals remains to be demonstrated, the recent finding of cecropinlike antimicrobial peptides in the porcine small intestine (25) suggests that the intestines may contain a variety of endogenous antimicrobial molecules. The further definition of such effector molecules could enhance our understanding of infections caused by pathogens that enter or affect the intestinal tract.

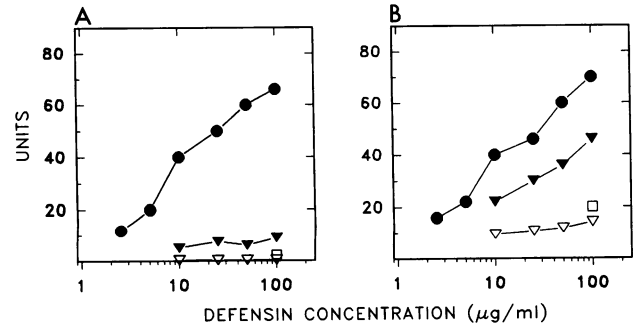

FIG. 9. Activities of cryptdins against *S. typhimurium*. Radial diffusion assays were performed at pH 7.4 against two smooth isogenic strains of *S. typhimurium*, 7953S (mouse avirulent, *phoP*) (B) and 14028S (mouse virulent, *phoP*$^+$) (A). Symbols: □, cryptdin 1; ▼, cryptdin 2; ●, NP-1; ▽, HNP-1. The control, 0.01% acetic acid, gave no zone of clearance.

ACKNOWLEDGMENTS

This work was supported by grants AI-29595 and AI-29839 from the National Institutes of Health and by a grant (001187-9-RG) to P. B. Eisenhauer from the American Foundation for AIDS Research.

We thank Alane Park, Nancy Chen, Lori Canning, and Demetrio Quismorio for skilled technical assistance. We also thank Audree Fowler for performing the amino acid sequence determinations at the UCLA Protein Microsequencing Facility, which is supported by BRS Shared Instrumentation grant 1S10RR05554 from the National Institutes of Health.

REFERENCES

1. **Ahonen, A., and A. Pentilla.** 1975. Effects of fasting and feeding and pilocarpine on Paneth cells of the mouse. Scand. J. Gastroenterol. **10**:347–352.
2. **Boman, H. G., and D. Hultmark.** 1987. Cell-free immunity in insects. Annu. Rev. Microbiol. **41**:103–126.
3. **Carter, P. B., and F. M. Collins.** 1974. The route of enteric

infection in normal mice. J. Exp. Med. **139:**1189–1203.
4. **Charp, P. A., W. G. Rice, R. L. Raynor, E. Reimund, J. M. Kinkade, Jr., T. Ganz, M. E. Selsted, R. I. Lehrer, and J. Kuo.** 1988. Inhibition of protein kinase C by defensins, antibiotic peptides from human neutrophils. Biochem. Pharmacol. **37:**951–956.
5. **Cheng, H.** 1974. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. IV. Paneth cells. Am. J. Anat. **141:**521–536.
6. **Deckx, R. J., G. R. Vantrappen, and M. M. Parein.** 1967. Localization of lysozyme activity in a Paneth cell granule fraction. Biochim. Biophys. Acta **139:**204–209.
7. **Diamond, G., M. Zasloff, H. Eck, M. Brasseur, W. L. Maloy, and C. L. Bevins.** 1991. Tracheal antimicrobial peptide, a novel cysteine-rich peptide from mammalian tracheal mucosa: isolation and cloning of a cDNA. Proc. Natl. Acad. Sci. USA **88:**3952–3956.
8. **Dickman, M. D., A. R. Chappelka, and R. W. Schaedler.** 1976. The microbial ecology of the upper small bowel. Am. J. Gastroenterol. **65:**57–62.
9. **Dubos, R. J., D. C. Savage, and R. W. Schaedler.** 1967. The indigenous flora of the gastrointestinal tract. Dis. Colon Rectum **10:**23–34.
10. **Eisenhauer, P. B., S. S. L. Harwig, D. Szklarek, T. Ganz, and R. I. Lehrer.** 1990. Polymorphic expression of defensins in neutrophils from outbred rats. Infect. Immun. **58:**3899–3902.
11. **Erlandsen, S. L., and D. G. Chase.** 1972. Paneth cell function: phagocytosis and intracellular digestion of spiral microorganisms. I. Hexamita muris. J. Ultrastruct. Res. **41:**296–318.
12. **Erlandsen, S. L., and D. G. Chase.** 1972. Paneth cell function: phagocytosis and intracellular digestion of spiral microorganisms. II. Spiral microorganisms. J. Ultrastruct. Res. **41:**319–333.
13. **Fields, P. I., E. A. Groisman, and F. Heffron.** 1989. A Salmonella locus that controls resistance to microbicidal proteins from phagocytic cells. Science **243:**1059–1062.
14. **Fleming, A.** 1932. Lysozyme. President's Address. Proc. R. Soc. Med. **26:**71–84.
15. **Ganz, T., J. R. Rayner, E. V. Valore, A. Tumolo, K. Talmadge, and F. Fuller.** 1989. The structure of the rabbit macrophage defensin genes and their organ-specific expression. J. Immunol. **143:**1358–1365.
16. **Ganz, T., M. E. Selsted, and R. I. Lehrer.** 1990. Defensins. Eur. J. Hematol. **44:**1–8.
17. **Gorbach, S. L.** 1971. Progress in gastroenterology. Intestinal microflora. Gastroenterology **60:**1110–1129.
18. **Groisman, E. A., E. Chiao, C. J. Lipps, and F. Heffron.** 1989. *Salmonella typhimurium phoP* virulence gene is a transcriptional regulator. Proc. Natl. Acad. Sci. USA **86:**7077–7081.
19. **Hammer, M. F., J. W. Schilling, E. M. Prager, and A. C. Wilson.** 1987. Recruitment of lysozyme as a major enzyme in the mouse gut. Duplication, divergence and regulatory evolution. J. Mol. Evol. **24:**272–279.
20. **Hammer, M. F., and A. C. Wilson.** 1987. Regulatory and structural genes for lysozymes of mice. Genetics **115:**521–533.
21. **Harwig, S. S. L., A. S. K. Park, and R. I. Lehrer.** Characterization of defensin precursors in mature human neutrophils. Blood, in press.
22. **Jolles, P., and J. Jolles.** 1984. What's new in lysozyme research. Mol. Cell. Biochem. **63:**165–189.
23. **Keshav, S., L. Lawson, L. P. Chung, M. Stein, V. H. Perry, and S. Gordon.** 1990. Tumor necrosis factor mRNA localized to Paneth cells of normal intestinal epithelium by in situ hybridization. J. Exp. Med. **171:**327–332.
24. **Lawson, A. J., R. A. Smit, N. A. Jeffers, and J. W. Osborne.** 1982. Isolation of rat intestinal crypt cells. Cell Tissue Kinet. **15:**69–80.
25. **Lee, J.-Y., A. Boman, S. Chuanxin, M. Andersson, H. Jornvall, V. Mutt, and H. G. Boman.** 1989. Antibacterial peptides from the pig intestine: isolation of a mammalian cecropin. Proc. Natl. Acad. Sci. USA **86:**9159–9162.
26. **Lehrer, R. I., A. Barton, and T. Ganz.** 1988. Concurrent assessment of inner and outer membrane permeabilization and bacteriolysis in E. coli by multiple-wavelength spectrophotometry. J. Immunol. Methods **108:**153–158.
27. **Lehrer, R. I., and T. Ganz.** 1990. Antimicrobial polypeptides of human neutrophils. Blood **76:**2169–2181.
28. **Lehrer, R. I., M. Rosenman, S. S. L. Harwig, R. Jackson, and P. Eisenhauer.** 1991. Ultrasensitive assays for endogenous antimicrobial polypeptides. J. Immunol. Methods **137:**167–173.
29. **Lichtenstein, A. K., T. Ganz, T. M. Nguyen, M. E. Selsted, and R. I. Lehrer.** 1988. Mechanism of target cytolysis by peptide defensins. Target cell metabolic activities, possibly including endocytosis, are critical for expression of cytotoxicity. J. Immunol. **140:**2686–2694.
30. **Lopez-Llewellyn, J., and S. L. Erlandsen.** 1980. Cytodifferentiation of the rat Paneth cell: an immunocytochemical investigation in suckling and weanling animals. Am. J. Anat. **158:**285–297.
31. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262:**10035–10038.
32. **Merzel, J.** 1967. Some histophysiological aspects of Paneth cells of mice as shown by histochemical and radioautographical studies. Acta Anat. **66:**603–630.
33. **Metchnikoff, E.** 1907. Immunity in infectious diseases, p. 423–424. Cambridge University Press, Cambridge.
34. **Miller, S. I., W. S. Pulkkinen, M. E. Selsted, and J. J. Mekalanos.** 1990. Characterization of defensin resistance phenotypes associated with mutations in the *phoP* virulence regulon of *Salmonella typhimurium*. Infect. Immun. **58:**3706–3710.
35. **Miller, S. J., A. M. Kukral, and J. J. Mekalanos.** 1989. A two-component regulatory system (*pho*P *pho*Q) controls *Salmonella typhimurium* virulence. Proc. Natl. Acad. Sci. USA **86:**5054–5058.
36. **Mushin, R., and R. Dubos.** 1965. Colonization of the mouse intestine with Escherichia coli. J. Exp. Med. **122:**745–757.
37. **Ouellette, A. J., R. M. Greco, M. James, D. Frederick, J. Naftilan, and J. T. Fallon.** 1989. Developmental regulation of cryptdin, a corticostatin/defensin precursor mRNA in mouse small intestinal crypt epithelium. J. Cell Biol. **108:**1687–1695.
38. **Poulsen, S. S., E. Nexo, P. Skav Olsen, J. Hess, and P. Kierkegaard.** 1986. Immunochemical localization of epidermal growth factor in rat and man. Histochemistry **85:**389–394.
39. **Pulkkinen, W. S., and S. I. Miller.** 1991. A *Salmonella typhimurium* virulence protein is similar to a *Yersinia enterocolitica* invasion protein and a bacteriophage lambda outer membrane protein. J. Bacteriol. **173:**86–93.
40. **Sahara, N., K. Fukasawa, M. Harada, and K. Suzuki.** 1983. Immunohistochemical localization of dipeptidyl peptidase IV in rat digestive organs. Acta Histochem. Cytochem. **16:**494–501.
41. **Satoh, Y.** 1988. Atropine inhibits the degranulation of Paneth cells in ex-germ-free mice. Cell Tissue Res. **253:**397–402.
42. **Satoh, Y.** 1988. Effect of live and heat-killed bacteria on the secretory activity of Paneth cells in germ-free mice. Cell Tissue Res. **251:**87–93.
43. **Satoh, Y., K. Ishikawa, H. Tanaka, and K. Ono.** 1986. Immunohistochemical observations of immunoglobulin A in the Paneth cells of germ-free and ex-germ-free rats. Histochemistry **85:**197–201.
44. **Satoh, Y., K. Ishikawa, H. Tanaka, Y. Oomori, and K. Ono.** 1988. Immunohistochemical observations of lysozyme in the Paneth cells of specific-pathogen-free and germ-free mice. Acta Histochem. **83:**185–188.
45. **Satoh, Y., M. Yamano, M. Matsuda, and K. Ono.** 1990. Ultrastructure of Paneth cells in the intestine of various mammals. J. Electron Microsc. Tech. **16:**69–80.
46. **Savage, D. C., R. Dubos, and R. W. Schaedler.** 1968. The gastrointestinal epithelium and its autochthonous flora. J. Exp. Med. **127:**67–76.
47. **Schaedler, R. W.** 1973. Symposium on gut microflora and nutrition in the nonruminant. The relationship between the host and its intestinal microflora. Proc. Nutr. Soc. **32:**41–47.
48. **Schagger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166:**368–379.

49. **Senegas-Balas, F., D. Balas, R. Verger, A. deCaro, C. Figarella, F. Ferrato, P. Lechene, C. Bertrand, and A. Ribet.** 1984. Immunohistochemical localization of intestinal phospholipase A2 in rat Paneth cells. Histochemistry **81**:581–584.
50. **Sheu, M. J., W. W. Baldwin, and K. W. Brunson.** 1985. Cytotoxicity of rabbit macrophage peptides MCP-1 and MCP-2 for mouse tumor cells. Antimicrob. Agents Chemother. **28**:626–629.
51. **Spicer, S. S., M. W. Staley, M. G. Wetzel, and B. K. Wetzel.** 1967. Acid mucosubstances and basic protein in mouse Paneth cells. J. Histochem. Cytochem. **15**:225–242.
52. **Territo, M. C., T. Ganz, M. E. Selsted, and R. I. Lehrer.** 1989. Monocyte-chemotactic activity of defensins from human neutrophils. J. Clin. Invest. **84**:2017–2020.
53. **Trier, J. S., V. Lorenzsonn, and K. Groehler.** 1967. Pattern of secretion of Paneth cells of the small intestine of mice. Gastroenterology **53**:240–249.
54. **Valore, E. V., and T. Ganz.** Posttranslational processing of defensins in immature human myeloid cells. Blood, in press.
55. **Weiser, M. M.** 1973. Intestinal epithelial cell surface membrane glycoprotein synthesis. I. An indicator of cellular differentiation. J. Biol. Chem. **248**:2536–2541.
56. **Wheeler, E. J., and J. K. Wheeler.** 1964. Comparative study of Paneth cells in vertebrates. Anat. Rec. **148**:350.
57. **Wray, W., T. Boulikas, V. P. Wray, and R. Hancock.** 1981. Silver staining of proteins in polyacrylamide gels. Anal. Biochem. **118**:197–203.
58. **Zasloff, M.** 1987. Magainins, a class of antimicrobial peptides from *Xenopus* skin: characterization of two active forms and partial cDNA sequence of a precursor. Proc. Natl. Acad. Sci. USA **84**:5449–5453.
59. **Zhu, Q. Z., J. Hu, S. Mulay, F. Esch, S. Shimasaki, and S. Solomon.** 1988. Isolation and structure of corticostatin peptides from the rabbit fetal and adult lung. Proc. Natl. Acad. Sci. USA **85**:592–596.
60. **Zhu, Q. Z., A. V. Singh, A. Bateman, F. Esch, and S. Solomon.** 1987. The corticostatic (anti-ACTH) and cytotoxic activity of peptides isolated from fetal, adult and tumor-bearing lung. J. Steroid Biochem. **27**:1017–1022.