Enteric Defensins: Antibiotic Peptide Components of Intestinal Host Defense

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Abstract. Five intestinal defensins, termed cryptdins 1–5, have been purified from mouse small bowel, sequenced, and localized to the epithelium by immunohistochemistry. Although identified as members of the defensin peptide family by peptide sequencing, enteric defensins are novel in that four cryptdins have amino termini which are three to six residues longer than those of leukocyte-derived defensins. A fifth cryptdin is the first defensin to diverge from the previously invariant spacing of cysteines in the peptide structure. The most abundant enteric defensin, cryptdin-1, had antimicrobial activity against an attenuated phoP mutant of *Salmonella typhimurium* but was not active against the virulent wild-type parent. Immunohistochemical localization demonstrated that cryptdin-1, and probably cryptdins 2 and 3, occur exclusively in Paneth cells, where the peptides appear to be associated with cytoplasmic granules. Biochemical and immunologic analysis of the luminal contents of the small intestine suggest that cryptdin peptides are secreted into the lumen, similar to Paneth cell secretion of lysozyme. The presence of several enteric defensins in the intestinal epithelium, evidence of their presence in the lumen, and the antibacterial activity of cryptdin-1 suggest that these peptides contribute to the antimicrobial barrier function of the small bowel mucosa.

DEFENSINS are highly abundant antimicrobial peptide components of vertebrate neutrophil and macrophage granules (Lehrer et al., 1991*a*). These cationic 3–4 kD peptides exhibit broad-range antimicrobial activities against gram negative and gram positive bacteria, many fungi, and some enveloped viruses (Ganz et al., 1990). Evidence suggests that defensins exert their antibacterial effect by permeabilizing the cytoplasmic membrane of the target microorganism (Lehrer et al., 1989) by a mechanism that may involve the formation of ion channels (Kagan et al., 1990; Hill et al., 1991). Members of the defensin family have been identified previously in human, rabbit, guinea pig, and rat phagocytes (Lehrer et al., 1991*a*). The peptides are characterized by nine highly conserved amino acids, including six invariant cysteine residues which constitute a unique disulfide motif (Fig. 1; Selsted and Harwig, 1989). The three disulfides stabilize a tertiary conformation consisting predominantly of beta-sheet (Pardi et al., 1988; Hill et al., 1991). The highly ordered structure and the absence of helix make defensins unique among known antimicrobial peptides.

A role for defensin peptides in enteric host defense was suggested by the identification of an mRNA that was predicted to code for a defensin in Paneth cells of the mouse small intestine (Ouellette et al., 1989*a*). Paneth cells are granulated epithelial cells that reside at the base of intestinal crypts (Madara and Trier, 1987), and they derive from stem cells that also produce villus enterocytes (Cheng et al., 1969). In contrast to short-lived enterocytes which ascend the villus and exfoliate into the lumen within 2–3 d, Paneth cells mature below the proliferative zone and have apparent lifetimes of ~20 d (Troughton and Trier, 1969). Paneth cells synthesize proteins generally considered to be characteristic of leukocytes, including tumor necrosis factor (TNF) (Keshav et al., 1990) and lysozyme (Speece, 1964; Hammer et al., 1987). Since lysozyme may be secreted into the lumen of the small bowel under cholinergic stimulation (Peeters and Vantrappen, 1975), these cells may secrete additional antimicrobial factors, thereby restricting the colonization and invasion of the epithelium by bacteria.

An intestinal defensin mRNA, termed cryptdin mRNA, was detected exclusively in Paneth cells of the small bowel (Ouellette et al., 1989*a*). Cryptdin mRNA is one of several highly abundant low molecular weight intestinal mRNAs which code for small, cysteine-rich polypeptides that accumulate in mouse small bowel during its postnatal maturation (Ouellette and Cordell, 1988; Ouellette and Lualdi, 1990). Mouse cryptdin mRNA is predicted to code for a 93 amino acid protein that is similar to the deduced human and rabbit neutrophil defensin precursors (Daher et al., 1988; Ganz et al., 1989). The cryptdin gene, *Defcr*, in the proximal region of chromosome 8, shows conserved linkage homology with the human defensin gene(s) *DEF1* on 8p23 (Ouellette et al., 1989*b*; Sparkes et al., 1989). By homology with leukocyte defensins, processing of the deduced cryptdin precur-

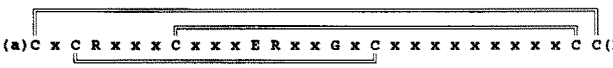

Figure 1. The covalent consensus structure of myeloid defensins. Residues which are invariant in 13 of 16 defensin peptides purified from neutrophils and macrophages are shown in single letter code. Residues which vary between defensins are shown as "x" characters; the "a" character represents 0–2 NH_2-terminal amino acid residues; the "b" character denotes 0–3 COOH-terminal amino acids. The disulfide bonding motif is depicted by connecting double lines.

sor was predicted to produce a peptide of 30–32 residues, depending upon the site of amino-terminal processing. To determine the actual cryptdin peptide sequence and to test for antibacterial activity, we isolated the predicted molecule. Here, we report the purification and sequencing of five enteric defensins, including cryptdin-1, the peptide predicted by the cryptdin cDNA sequence. In addition, we present data that demonstrate the antibacterial activity of the most abundant enteric defensin, immunohistochemical localization of one or more enteric defensins to Paneth cells, and immunochemical and biochemical evidence suggestive of secretion of defensins into the lumen of the small bowel.

Materials and Methods

Animals and Tissue Preparation

Outbred Swiss mice [(Crl:CD-1)(ICR)BR], 45-d-old males (30–35 g) or timed-pregnant dams, were obtained from Charles River Breeding Laboratories, Inc. (North Wilmington, MA). Mice were housed under 12-h cycles of light and darkness and had free access to food and water. Jejunum and ileum were removed en bloc from mice killed by cervical dislocation. The lumen of each resected intestine was rinsed with 35 ml PBS, and the wash was acidified by addition of 3.5 ml glacial acetic acid and frozen. Washed intestines from individual mice were disrupted thoroughly in 35-ml ice-cold 30% formic acid using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Homogenates were stirred continuously for 18 h at 4°C, clarified by centrifugation at 27,000 rpm in the SW28.1 rotor for 30 min at 4°C, lyophilized, and stored at −85°C.

Sheets of intestinal epithelium were isolated by EDTA perfusion (Bjerknes and Cheng, 1981). After irrigation of the intestinal lumen, anesthetized mice were perfused systemically with 30 mM EDTA in Ca^{++}/Mg^{++}-free HBSS by left ventricular injection. Epithelial sheets were separated from basement membrane of the underlying lamina propria by gentle shaking of the everted intestine in ice-cold Mg^{++}-free Hank's buffer. Under these conditions, sheets of intestinal epithelium were released from the lamina propria and then sedimented by centrifugation. The pelleted cells were then homogenized as before in 10 ml of 30% formic acid.

Purification of Enteric Defensins

Lyophilized acid extracts were dissolved in 100 ml of 30% acetic acid, stirred for 2 h at room temperature, and clarified by centrifugation at 27,000 *g* at 22°C for 1 h. The supernatant was concentrated to 30 ml by centrifugal evaporation and chromatographed (15 ml per loading) on a 2.5 × 55 cm column of Bio-Gel P-60 equilibrated in 30% acetic acid. 7.5-ml fractions were collected at 15 ml/h while the effluent was continuously monitored at 280 nm. A 200-μl sample of each fraction was lyophilized, dissolved in 30 μl of 5% acetic acid containing 3.0 M urea, and electrophoresed on 12.5% acid-urea acrylamide gels (Selsted and Harwig, 1987). Protein bands were visualized with Coomassie R-250.

Fractions containing putative defensins were identified by acid-urea PAGE, in which the peptides migrated rapidly (>0.6 × R_f of the methyl green tracking dye) and by SDS-PAGE where the peptides had apparent M_r's of 4. These fractions were pooled, lyophilized, and dissolved in 6 ml of 30% acetic acid and rechromatographed on Bio-Gel P-60. Final purification of five enteric defensins was achieved by RP-HPLC on a 0.46 × 25 cm Vydac C-18 column using HFBA and TFA as ion-pairing agents as described in Results.

Peptide Characterization

Amino acid analyses were performed on 6 N HCl hydrolysates (150°C, 2 h) of unmodified or performic-acid oxidized peptides. Hydrolysates were derivatized with phenylisothiocyanate, and the resulting phenythiocarbamyl amino acids were quantitated as described previously (Selsted and Harwig, 1987). Peptide samples were reduced with DTT and pyridylethylated with 4-vinylpyridine for sequencing (Henschen, 1986). Sequence determinations were performed by automated Edman degradation on an ABI model 477 system (Applied Biosystems, Inc., Foster City, CA) with on-line PTH amino acid analysis. In certain cases, the COOH terminus was confirmed by amino acid analysis of tryptic peptides.

Antimicrobial Assay

Antibacterial activity was measured in an agar diffusion assay (Lehrer et al., 1991*b*) using wild-type *Salmonella typhimurium* (ATCC10428) or an isogenic phoP mutant of *S. typhimurium* (strain CS015 phoP102::Tn10d-Cam; Miller et al., 1989). ATCC10428 and CS015 were grown to log phase in trypticase soy broth at 37°C, harvested by centrifugation, and resuspended to 1×10^7 colony forming units (CFU) per ml in 10 mM sodium phosphate buffer (pH 7.4). A 100-μl aliquot of each organism was mixed with 10 ml of 1% agarose in 0.03% (wt/vol) trypticase soy medium, 10 mM sodium phosphate (pH 7.4) at 42°C. 5-μl samples of peptide solution were pipetted into 3-mm diameter wells formed in the agarose with a sterile punch. After 3 h at 37°C, the inoculated agarose plate was overlaid with 1% agarose containing 6% trypticase soy medium. After 12–16 h, antimicrobial activity was apparent as clear zones surrounding wells loaded with antibacterial samples.

Anticryptdin Antibody

A polyclonal rabbit antibody was prepared to a synthetic analogue of cryptdin-1. The peptide, cryptdin-C, corresponding to residues 4–35 in cryptdin-1 (see Fig. 4) was synthesized by solid phase chemistry using N^{α}-butoxycarbonyl protection (Kent, 1988). After cleavage/deprotection of synthetic cryptdin-C with TFA-trifluoromethanesulfonic acid, the peptide was precipitated in ethyl ether and dried in vacuo. A 100-mg sample was dissolved in 10 ml of 6.0 M guanidine-HCl, 0.2 M Tris-HCl, pH 8.2, containing 20 mg of DTT. The sample was purged with nitrogen, heated to 50°C for 4 h, diluted 100-fold with deionized water, and dialyzed exhaustively, first against 0.1 M sodium phosphate (pH 8.2), 20 mM guanidine-HCl, 100 mM NaCl, then against 5% acetic acid. The sample was lyophilized, dissolved in 10 ml 5% acetic acid, and subjected to RP-HPLC on a 1 × 25 cm Vydac C-18 column. The earliest eluting peak, representing ~0.5% of the crude peptide was determined by amino acid analysis to have the desired composition.

A 1.5-mg sample of cryptdin-C was supplied to Berkeley Antibody Company (Berkeley, CA) for immunization of two New Zealand White rabbits. Serum samples were collected for 12 wk, until the anticryptdin C titer, determined by ELISA, reached ~1:10,000 for each rabbit. IgG was isolated from antiserum using DEAE Econo-Pac chromatography (Bio-Rad Laboratories, Richmond, CA) as described by the manufacturer.

Immunohistochemistry

Paraffin sections of formalin-fixed mouse mid small bowel were deparaffinized, treated with 1.1% hydrogen peroxide for 40 min, washed extensively with water and then with PBS. Slides were treated for 20 min at 37°C with 500 μg/ml trypsin in PBS, washed twice with PBS, and blocked for 20 min with 5% porcine serum. Slides were incubated for 20 min in rabbit anti-cryptdin IgG (1:10 dilution relative to serum IgG concentration), and washed with blocking serum. Porcine anti-rabbit IgG was used as linking reagent between the primary antibody and rabbit antiperoxidase–peroxidase conjugate (Dako, Carpenteria, CA). DAB was used as peroxidase substrate, and parallel incubations were performed using equivalent dilutions of rabbit preimmune IgG as the primary antibody.

Results

Purification of Enteric Defensins

Initial efforts to purify intestinal defensins focused on the isolation of cryptdin-1, the peptide predicted from the cryptdin cDNA sequence. Since the deduced peptide is highly cat-

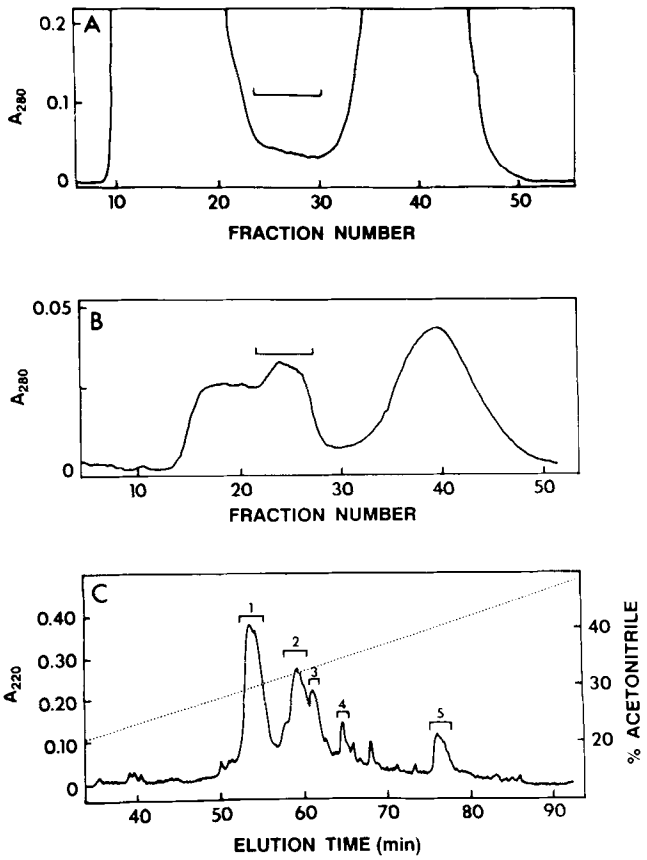

Figure 2. Purification of enteric defensins. Acid extract of jejunum and ileum was chromatographed in 30% acetic acid on a P-60 column. Fractions indicated by brackets (*A*) were pooled and rechromatographed on the P-60 column (*B*). Defensin-containing fractions (*brackets*, *B*) were pooled and further purified by RP-HPLC on a 0.46 × 25 cm Vydac C-18 column. Water-acetonitrile gradient elution (*dashed line*) using 0.13% (vol/vol) HFBA as modifier was used to purify cryptdins 1–5. The brackets in *C* indicate the peptide contained in each peak, and the portion of each which was subjected to a second round of RP-HPLC.

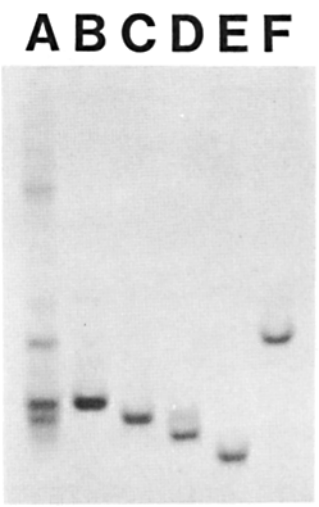

Figure 3. Acid urea PAGE of enteric defensins. Samples of low molecular weight enteric peptides obtained by P-60 gel filtration (Fig. 2, *B*, *brackets*) and purified cryptdins were electrophoresed on a 12.5% acid-urea gel, and stained with formalin-containing Coomassie blue. (Lane *A*) ~20 μg P-60 low molecular weight peptide fraction; (*B–F*) 1 μg each of cryptdins 1–5, respectively.

ionic, intestinal peptides were solubilized by homogenizing intact jejunum and ileum in 30% formic acid. Acid-urea PAGE of the crude extract revealed several bands with R_f values similar to those of rabbit defensin NP-1 and cryptdin-C (data not shown), a folded synthetic defensin congener corresponding to residues 4–35 in cryptdin-1 (Material and Methods). Peptides corresponding to these bands were purified ~200-fold by two rounds of gel filtration chromatography on Bio-Gel P-60 (Fig. 2, *upper* and *middle*). Electrophoresis of P-60 column fraction samples on acid-urea gels showed that five fractions which eluted between two prominent peaks (Fig. 2, *A* and *B*, *brackets*) contained putative cryptdin peptides (Fig. 3, lane *a*). Peptides in these P-60 fractions migrated with M_r's of ~4 on SDS-PAGE (data not shown), consistent with the molecular weight of defensins. Furthermore, treatment of P-60 fraction samples with performic acid markedly reduced the electrophoretic mobility of the five putative cryptdins in acid-urea gels (data not shown), behavior that is characteristic of defensins and polypeptides that contain multiple cysteine residues.

Defensins in pooled P-60 fractions were purified further using sequential rounds of RP-HPLC utilizing different ion-pair agents. Initial HPLC fractionation utilized water–acetonitrile gradients containing 0.13% heptafluorobutyric acid (HFBA) as the ion-pairing agent, whereby each of the five peptides contained in the pooled P-60 fractions was resolved to near purity in a single run (Fig. 2, *bottom*). Complete purification of five peptides, cryptdins 1–5, was achieved by subsequent RP-HPLC using 0.1% trifluoroacetic acid (TFA) (Fig. 3, lanes *b–f*). Assuming that extraction of individual peptides was equally efficient, both acid-urea gel electrophoresis and RP-HPLC of the P-60 fractions containing putative cryptdins showed that the relative abundance of the peptides is cryptdin-1 > cryptdin-2 > cryptdin-5 > cryptdin-3 > cryptdin-4. These relative amounts have been qualitatively reproducible in every preparation of acid-extracted protein from mouse small intestine. We have not established whether differences in cryptdin levels results from variation between individual crypts, between individual mice, or differential levels of individual cryptdins along the length of the bowel.

Characterization of Cryptdins 1–5

Biochemical characterization of cryptdins 1–5 demonstrated that these peptides are defensins. Amino acid analysis of each peptide showed that their compositions were compatible with defensin-like molecules: cationic peptides of 31–35 residues which included 6 half-cystines (Table I). The complete sequences of cryptdins 1–5 were determined by automated degradation and amino acid analysis of carboxyl-terminal tryptic peptides. As shown in Fig. 4, the primary structures of the five enteric defensins contain the distinctive structural features of human, rabbit, rat, and guinea pig neutrophil defensins (Lehrer et al., 1991*a*), i.e., the six invariant cysteine residues, and glycine and glutamic acid in positions that are also highly conserved in myeloid defensins.

Cryptdins 1–5 contain features that are unique and distinct from defensins of myeloid origin. Cryptdin-1 is the peptide predicted by the cryptdin cDNA sequence, and it is almost identical to cryptdins 2 and 3, differing in sequence only at position 10 (Ser, Thr, or Lys), position 15 (Gly or Arg), or position 29 (Leu or Met) as shown in Fig. 4. Analysis of codons from which these amino acid differences could arise shows that the conversion of Ser^{10} to Lys^{10} in cryptdins 1 and 3, respectively, requires two nucleotide substitutions. On the other hand, single nucleotide changes in cryptdin-2 could give rise both to cryptdins 1 and 3, suggesting that the

Table I. Amino Acid Analysis of Cryptdins 1–5

Residue	Cryptdin-1	Cryptdin-2	Cryptdin-3	Cryptdin-4	Cryptdin-5
Cysteine	5.22 [6]	5.29 [6]	5.86 [6]	6.60 [6]	5.90 [6]
Aspartic acid	1.74 [2]	1.72 [2]	1.88 [2]	0	1.35 [1]
Glutamic acid	1.31 [1]	1.01 [1]	1.03 [1]	1.37 [1]	1.40 [1]
Serine	0.84 [1]	0	0	0	1.31 [1]
Glycine	4.28 [4]	2.73 [3]	2.92 [3]	4.80 [5]	2.09 [2]
Histidine	0.67 [1]	0.90 [1]	0.88 [1]	0.99 [1]	0
Arginine	7.04 [7]	7.76 [8]	8.35 [8]	5.98 [6]	6.16 [6]
Threonine	2.06 [2]	2.40 [3]	1.96 [2]	1.25 [1]	1.68 [2]
Alanine	0	0	0	0	0
Proline	0	0	0	0.87 [1]	0
Tyrosine	1.90 [2]	1.81 [2]	1.73 [2]	1.94 [2]	1.04 [1]
Valine	0.96 [1]	0.88 [1]	0.95 [1]	1.05 [1]	2.02 [2]
Methionine	0.62 [1]	1.78 [2]	1.68 [2]	0	0
Isoleucine	0	0	0	0.88 [1]	1.91 [2]
Leucine	4.10 [5]	4.50 [5]	4.21 [4]	3.21 [3]	3.77 [4]
Phenylalanine	0	0	0	0.85 [1]	3.63 [4]
Tryptophan	0	0	0	0	0
Lysine	1.97 [2]	2.07 [2]	2.83 [3]	2.12 [2]	2.87 [3]
Total	35	35	35	31	35

Brackets denote residue numbers determined by sequence analysis.

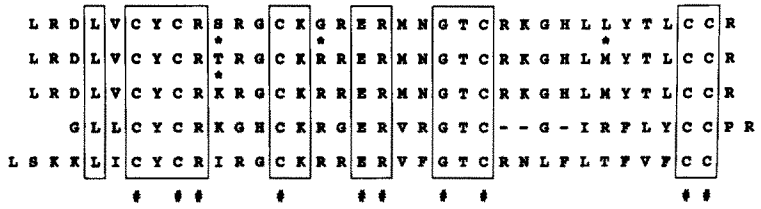

Figure 4. Amino acid sequences of five enteric defensins. Primary structures of cryptdins 1–5 are shown in single letter amino acid code. Primary structures are aligned to maximize sequence similarity, with hyphens denoting gaps in the cryptdin-4 sequence. The cryptdin consensus is indicated by the boxed residues. Differences in sequence between cryptdins 1–3 are indicated by "*" symbols between lines of peptide sequence. Residues of the myeloid defensin consensus are depicted by "#" characters.

cryptdin-2 gene may be an intermediate or progenitor of the cryptdin-1 and cryptdin-3 genes.

By homology with the structures of known myeloid defensins, the cryptdin-1 NH_2 terminus had been predicted to be at Leu[4] or Val[5], 1 or 2 residues before the first cysteine. However, compared to myeloid defensins, intestinal defensins have variably extended NH_2 termini that contain from three (cryptdin-4) to six (cryptdin-5) amino acids preceding the first cysteine. In cryptdins 1–3 and 5, the N-peptidyl extensions consist of two charged internal residues flanked by amino acids with hydrophobic sidechains. Since natural variation in defensin amino termini has been shown to correlate with relative antimicrobial potency in vitro (Ganz et al., 1985), we speculate that the extended NH_2 termini of enteric defensins may have evolved for a unique role in the bowel or that the peptides undergo further processing at the amino terminus.

Cryptdin-4, the most cathodal (and apparently least abundant) enteric defensin is the first defensin found to contain a chain length variation between the fourth and fifth cysteine residues. Unlike all known defensins in which nine amino acids separate the fourth and fifth cysteines (Lehrer et al., 1991*a*), cryptdin-4 contains only six residues between the same two amino acids. This finding reveals that the defensin fold involving this stretch of the peptide chain can accommodate a substantially smaller loop than the one defined by crystal and NMR structures, respectively, of human and rabbit neutrophil defensins (Hill et al., 1991; Pardi et al., 1988).

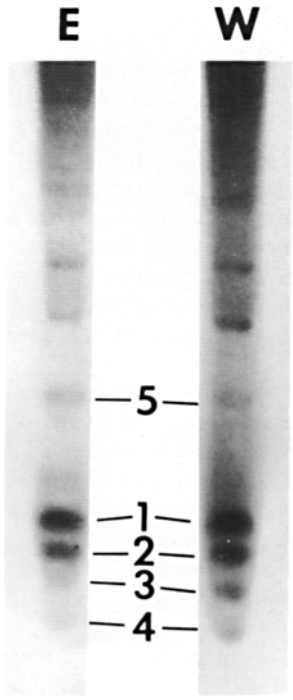

Figure 5. Identification of cryptdins 1–5 in small intestinal epithelium. Acid extracts of intact, whole small intestine (*W*) or epithelial sheets were lyophilized, dissolved in sample solution and resolved on a 12.5% acid-urea acrylamide gel. Cryptdins 1–5 are identified numerically.

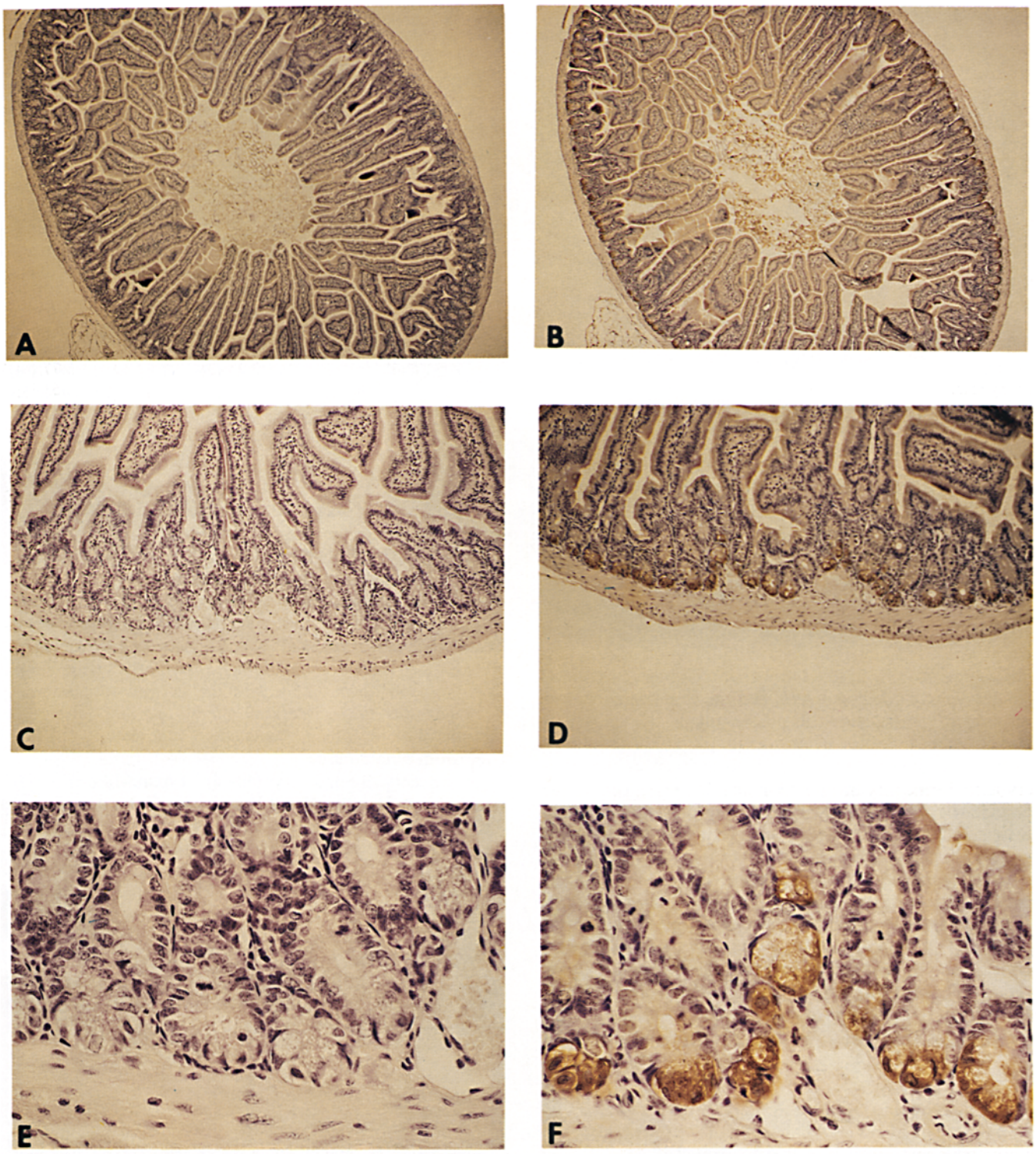

Figure 6. Immunohistochemical localization of cryptdin-1 in small intestine. Full thickness sections of adult mouse jejunem were incubated with preimmune (*A*, *C*, *E*) or anti-cryptdin-C rabbit IgG (*B*, *D*, *F*) and developed using peroxidase-antiperoxidase secondary antibody. Original magnifications: *A* and *B*, 40X; *C* and *D*, 250X; *E* and *F*, 640X.

Cryptdins 1–5 Are Epithelial Defensins

Cryptdins 1–5 derive from intestinal epithelial cells. In the presence of EDTA, the intestinal epithelium no longer adheres to the underlying basement membrane and floats free of the lamina propria upon gentle agitation (Bjerknes and Cheng, 1981). Preparations of epithelial sheets isolated in this manner (Materials and Methods) were concentrated by low-speed centrifugation and extracted with 30% formic acid. Peptides extracted from isolated epithelial sheets co-migrated with cryptdins 1–5 when analyzed by acid-urea PAGE (Fig. 5), demonstrating their epithelial origin.

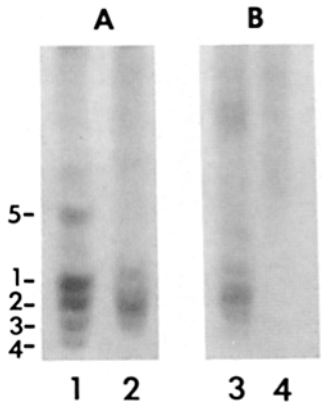

Figure 7. Comparison of cryptdins 1–5 and partially purified luminal peptides. (*A*) Lyophilized luminal lavage of small intestine from 12 mice was fractionated by P-60 gel filtration (Materials and Methods) and electrophoresed on an acid-urea acrylamide gel (20 μg; lane *2*) along side a similarly prepared sample of bowel tissue extract (20 μg; lane *1*). The positions of cryptdins 1–5 are indicated; (*B*) Partially purified luminal peptides (20 μg; same material as in lane *2*) was electrophoresed in a second acid-urea gel (lane *3*) along with an identical sample which had been previously treated with performic acid (lane *4*). In lane 4, rapidly migrating, cyst(e)ine-containing peptides are absent due to the increased net negative charge resulting from the conversion of cyst(e)ines to cysteic acid residues.

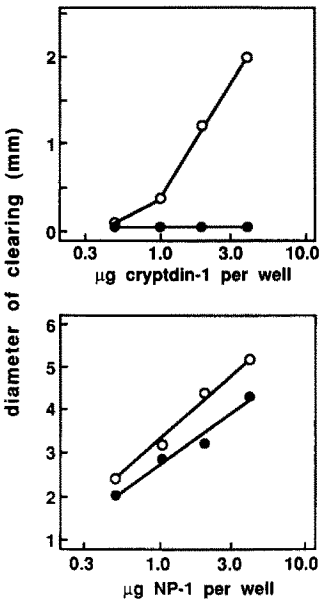

Figure 8. Antimicrobial activity of mouse cryptdin-1. Samples of rabbit NP-1 or purified natural cryptdin-1 dissolved in 0.01% acetic acid were pipetted into wells produced in a 0.6% agarose/0.3% tryptone plate containing 1×10^6 log phase cells. After incubation at 37°C for 18 h, antimicrobial activity was evaluated by measurement of the diameters of the clear zones. Closed circles denote wild-type *S. typhimurium*; open circles denote the phoP mutant.

Immunohistochemical Localization of Cryptdin-1 in Small Bowel

Immunoperoxidase staining of full-thickness sections of small intestine with an anticryptdin antibody demonstrated cryptdin antigen in Paneth cells, consistent with localization of cryptdin mRNA by in situ hybridization (Ouellette et al., 1989*a*). Incubation of sections of adult mouse jejunum and ileum with a polyclonal anti-cryptdin IgG produced by rabbits immunized with the synthetic congener cryptdin-C (Materials and Methods) localized the immunoperoxidase reaction to granulated cells, morphologically defined as Paneth cells, at the base of every crypt (Fig. 6). The staining pattern accentuates the granular appearance of the cytoplasm in these cells, and the immunoreactivity appears to be particularly strong over Paneth cell granules. The antibody is specific for mouse cryptdin(s), since it is negative both for rat and human Paneth cells (data not shown). Leukocytes in the lamina propria of the villi also were negative, suggesting that related enteric defensins are not expressed by phagocytes or lymphocytes. Because of the extensive similarity of cryptdins 1-3 (Fig. 4), the polyclonal antibody produced against cryptdin-C probably recognizes the three peptides. Conversely, because cryptdins 4 and 5 differ markedly from cryptdins 1-3, the anti-cryptdin-C antibody is unlikely to react with cryptdins 4 and 5 leaving their origin in Paneth cells somewhat unresolved.

Cryptdins in the Intestinal Lumen

Immunohistochemical and biochemical data suggest that cryptdins are present in the intestinal lumen. In each of several independently processed full section preparations, material in the small intestinal lumen was strongly immunoperoxidase-positive with anti-cryptdin-C antibody, but negative for preimmune sera or IgG. The reproducible pattern of immunoreactivity in the lumen is well represented by the photomicrographs in Figs. 6 (*A* and *B*). Consistent with immunochemical detection of anti-cryptdin-C positive material in the intestinal lumen, peptides in saline washes of adult jejunum and ileum eluted from the P-60 column at the same elution volume as cryptdins 1–5 and in acid-urea PAGE have mobilities similar to those of cryptdins 1–4. (Fig. 7 *A*). Though the lavage peptides are not identical to the characterized cryptdins in acid-urea PAGE (Fig. 7 *A*) or RP-HPLC analysis (data not shown), the fact that they are small and cationic is demonstrated by their electrophoretic migration (Fig. 7), and their disappearance from acid-urea gels when treated with performic acid (which occurs with treatment of all defensins) indicates that they are cysteine-rich (Fig. 7 *B*). Taken together, these data suggest that cryptdin-related peptides may exist in the intestinal lumen, and that these peptides may be processed variants of the enteric defensins isolated from whole tissue.

Conceivably, luminal cryptdin or cryptdin-like material could derive from exfoliated Paneth cells in the lumen, but the low rate of Paneth cell turnover suggests that is unlikely. Since lysozyme, another protein constituent of Paneth cell granules, is secreted into the lumen under cholinergic stimulation (Peeters and Vantrappen, 1975), it is possible that similar stimuli are responsible for secretion of intestinal defensins. The release of cryptdins or processed variants into the small bowel by Paneth cells contrasts with the apparent lack of defensin secretion by leukocytes (Ganz, 1987), and we propose that a secretory pathway may exist for the constitutive delivery of defensins into the intestinal lumen by Paneth cells.

Antimicrobial Activity of Cryptdin-1

The antibacterial activity of purified cryptdin-1, the most abundant enteric defensin, was tested against wild-type and phoP mutant *S. typhimurium* using a modified plate diffusion assay (Lehrer et al., 1991*b*). phoP is a two-component regulatory locus that is essential to *S. typhimurium* virulence and survival within macrophages (Fields et al., 1989; Miller et al., 1989), and mutants in the locus are particularly sensitive to rabbit defensins NP-1 and NP-2 when compared to wild-type parent strains (Fields et al., 1989; Miller et al., 1990). Under the assay conditions described (Material and Methods), the antimicrobial activity of rabbit defensin NP-1 against wild-type and the phoP mutant organisms is quite similar (Fig. 8, lower panel). On the other hand, wild-type

S. typhimurium is completely resistant to the effects of cryptdin-1 at concentrations of the peptide that are effective against the attenuated mutant (Fig. 8, *upper*). The differential activity of cryptdin-1 against avirulent *S. typhimurium* suggests that resistance to mucosal defensins may be of particular importance for the evolution of virulence in enteric pathogens.

Discussion

Before the characterization of a mouse intestinal defensin cDNA, expression of defensins was thought to be limited to professional phagocytes, i.e., neutrophils and macrophages. The presence of high levels of cryptdin mRNA in Paneth cells led to the hypothesis that defensins synthesized in intestinal epithelium may contribute to antimicrobial barrier function in the small bowel (Ouellette et al., 1989*a*). Our isolation and characterization of five cryptdin peptides and the demonstration of antibacterial activity of the most abundant peptide, cryptdin-1, provides additional evidence for the antimicrobial role of defensins in the small intestine. The immunohistochemical localization of cryptdin(s) to Paneth cells is consistent with previous in situ hybridization analysis, and suggests that defensins produced by these cells may act to limit bacterial colonization and invasion of the small bowel.

To establish the epithelial origin of cryptdins 1–5, we demonstrated their presence in sheets of intestinal epithelium which were free of underlying lamina propria. Acid-urea PAGE analysis of epithelial sheet extracts showed that the five enteric defensins were present in approximately the same relative quantities observed in the whole organ extracts (Fig. 5). The epithelial sheet preparations were judged to be devoid of stromal elements, because sheets prepared from 21-d-old mice by the same method lack class II antigen–associated invariant chain mRNA even though the lamina propria from the same mice is strongly positive for this mRNA (Sanderson et al., 1992).

To localize enteric defensins by immunohistologic methods, an antibody was raised in rabbits immunized with a synthetic peptide containing residues 4–35 of cryptdin-1, termed cryptdin-C (Materials and Methods). Correct folding and disulfide bond formation is essential to obtaining antibodies to natural or synthetic defensins (Selsted, M. E., unpublished data). Similarly, only correctly folded and oxidized synthetic cryptdin-C was sufficiently immunogenic to elicit IgG that was immunologically reactive with cryptdin-1 in an ELISA and in tissue sections; antibodies raised against linear cryptdin peptide fragments were immunologically unreactive. As noted above, because cryptdins 1–3 are identical at 32 of 35 residue positions, it is likely that the anti-cryptdin-C antibody reacts with all three peptides. To date, Western immunoblot analysis of individual cryptdins has not been possible, perhaps because the antibody-binding epitope is masked or altered by association with the membrane matrix.

Immunohistochemical analysis localized cryptdin antigen to the cytoplasm of Paneth cells of every crypt, and within these cells the staining was particularly strong over granules (Fig. 6 *C*). In addition, cryptdin antigen appears to be present in the bowel lumen (Fig. 6). Though the immunoperoxidase "background" appears increased in sections developed with anti-cryptdin-C antibody, the darker staining is predominantly surface associated. We suggest that this may reflect the presence of cryptdin antigen coating the mucosal surface. Based on these observations, we speculate that Paneth cells secrete defensins into the space above the crypt, where they may contribute to establishment of a local antibacterial milieu. This would be consistent with the ability of Paneth cells to secrete lysozyme in response to cholinergic agents (Staley and Trier, 1965; Peeters and Vantrappen, 1975; Satoh et al., 1989).

Peptides detected and partially purified from luminal lavage appear to be cryptdins that have been modified as compared to those isolated from intact tissue (Fig. 7). Possibly, the NH_2-terminal extensions that characterize cryptdins 1–3 undergo further processing in conjunction with their secretion. Active secretion of intestinal defensins would distinguish them from phagocyte defensins, which are not normally secreted and appear to be primarily targeted for intracellular delivery to the phagolysosome (Ganz, 1987). Experiments are underway to determine whether mouse enteric defensins are secreted in response to cholinergic stimuli, and the structural relationships of the tissue-extracted and luminal forms.

Enteric pathogens require the ability to resist mechanisms of mucosal immunity in order to cause disease. *S. typhimurium* is a highly virulent enteric pathogen of mice which causes typhoid fever. Strains of *S. typhimurium* with mutations in the phoP locus, are avirulent and simultaneously acquire increased sensitivity to neutrophil defensins (Fields et al., 1989). This locus has been characterized as a two component regulatory system consisting of two genes, phoP and phoQ, which exert transcriptional control over the synthesis of proteins that are essential to virulence (Miller, 1991; Miller et al., 1989; Pulkkinen and Miller, 1991). When cryptdin-1 was assayed for activity against the wild-type and phoP mutant strains of *S. typhimurium*, dose-dependent clearing was observed against the mutant only, in contrast to the result with rabbit neutrophil defensin NP-1 (Fig. 8). The susceptibility or resistance of isogenic Salmonella strains to Paneth cell antibacterial peptides may provide a system for studying pathogen–host interactions in vitro. *S. typhimurium* may have evolved virulence factors that allow for survival and invasion in an intestinal environment rich in enteric defensins.

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Note Added in Proof. A report summarizing an earlier method for purification and sequencing of cryptdin-1 was recently published (Ouellette, A. J., S. I. Miller, A. H. Henschen, and M. E. Selsted. 1992. Purification and primary structure of murine cryptdin-1, a Paneth cell defensin. *FEBS [Fed. Eur. Biochem. Soc.] Lett.* 304:146–148).

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