INFECTION AND IMMUNITY, June 1997, p. 2389–2395 Vol. 65, No. 6
0019-9567/97/$04.00+0


# Localization of Human Intestinal Defensin 5 in Paneth Cell Granules

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Received 27 September 1996/Returned for modification 25 November 1996/Accepted 5 March 1997

**Antibiotic peptides of higher animals include the defensins, first discovered in phagocytic cells but recently also found to be produced by epithelial cells. We biosynthesized recombinant human intestinal defensin 5 (rHD-5) using the baculovirus-insect cell expression system. Since insect cells process defensin incompletely and secrete the precursor proHD-5, we substituted a methionine for an alanine at a likely processing site to allow selective chemical cleavage with cyanogen bromide, and rHD-5 was used to elicit polyclonal antibodies. By the immunoperoxidase-staining technique, the antibodies selectively stained Paneth cells of the normal adult small intestine. Immunogold electron microscopy further localized HD-5 to the Paneth cell secretory granules. Since some defensins exert activity cytotoxic to mammalian cells, we assayed the effect of rHD-5 on the human intestinal cell lines Caco2 and Int407. proHD-5 did not exert cytotoxic activity, and rHD-5 showed only minimal activity against Int407 and was inert against Caco2. Since Paneth cells release their granules adjacent to the mitotic cells of the intestinal crypts, HD could protect this cell population against invasion and parasitization by microbes.**

Antimicrobial proteins and peptides are a major component of the host defense arsenal of phagocytes (39). In the neutrophils of humans and many vertebrates, the most abundant of the antimicrobial peptides are the defensins, a family of 3- to 4-kDa cationic, arginine- and cysteine-rich peptides with two subfamilies, α and β, that differ in their disulfide-bonding patterns (22). Recent studies have revealed that defensins are also expressed in epithelial cells. In the mouse small intestine, Paneth cells secrete cryptdins, α, or classical, defensins with documented microbicidal activity (16, 54, 69). In cattle, the mucosal β-defensins lingual antibiotic peptide and tracheal antibiotic peptide (15, 67) have been shown to be induced by trauma and microbial products (57). Most recently, human β-defensin 1 (6) was localized to the kidneys and female reproductive tract epithelium (77). Human Paneth cells also express defensins (human intestinal defensins 5 [HD-5] and HD-6) as determined by in situ hybridization (31, 32); however, the corresponding peptides have not been previously isolated and characterized. Paneth cells are multifaceted cells (8, 24, 56, 62), which have many features similar to those of myeloid cells. They contain granules with lysozyme (35, 44, 58) and other antimicrobial factors (29, 49, 63) which are released upon microbial challenge (45), and they express tumor necrosis factor alpha (TNF-α) (5, 66, 74), CD1 (37), and CD15 (4).

We wished to study the distribution and biological activity of human Paneth cell defensins. In this study, we prepared HD-5 in recombinant baculovirus, produced anti-recombinant HD-5 (rHD-5) polyclonal antibodies, and used them to localize naturally occurring HD-5 at the tissue and subcellular levels. We also assayed the cytotoxicity of rHD-5 to intestinal cell lines. In a subsequent study we describe the antimicrobial spectrum of rHD-5 under conditions simulating the intestinal milieu (55a).

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## MATERIALS AND METHODS

**Construction of recombinant proHD-5 baculovirus.** To avoid cumbersome purification from scarce specimens of fresh human intestine and to obtain adequate amounts of HD-5 for in vitro studies, HD-5 was biosynthesized (rHD-5). The baculovirus expression system (Clontech, Palo Alto, Calif.) was used to overexpress the HD-5 precursor preproHD-5 in insect *Spodoptera frugiperda* Sf21 cells, as described previously for human neutrophil defensin 1 (HNP-1) (78). HD-5 cDNA was kindly provided by Charles Bevins (31). Flanking *Bam*HI restriction sites for cloning of HD-5 cDNA into the transfer vector were introduced by PCR mutagenesis. Since insect cells process preprodefensin incompletely and secrete prodefensin (78), we introduced into the HD-5 cDNA a methionine substitution at a likely processing site (proHD5$^{Met}$) (Fig. 1) to permit selective chemical cleavage with cyanogen bromide (CNBr). The required mutations in the cDNA were generated by PCR-directed mutagenesis with four primers (A [HD-5 sense], 5′-CTCTGGATCCTGCAGGTGACCCCAGCCATG AGG; B [methionine substitution antisense], 5′-GCAATAGCAGGTCATTCT TGCCTGA; C [methionine substitution sense], 5′-CAGGCAAGAATGACCT GCTATTGC; and D [HD-5 antisense], 5′-TTTGGATCCTATCTAGGAAGC). PCRs were carried out with *Pfu* polymerase (Stratagene, La Jolla, Calif.), as specified by the manufacturer. Purification of PCR products was done by electrophoresis in a low-melting-point agarose gel. The bands with the appropriate sizes were excised from the gel and purified with the Prep-A-Gene kit (Bio-Rad, Richmond, Calif.) according to the manufacturer's instructions. The DNA fragments coding for the 5′ *Bam*HI restriction site-3′ methionine substitution (primers A and B) and 5′ methionine substitution-3′ *Bam*HI restriction site (primers C and D) were generated by PCR, purified, and then used as templates in a subsequent overlap extension reaction (30, 76) with primers A and D. The resulting cDNA fragment contained the ribosomal binding site, the translation start codon ATG, the entire coding region of preproHD-5$^{Met}$, a stop codon, and, at the 5′ and 3′ ends, *Bam*HI restriction sites. The fragment was purified, subjected to *Bam*HI digestion, ligated into the pBacPAK1 transfer vector, and transformed into *Escherichia coli* XL-1 Blue (13). Defensin-containing clones were identified by detection of the insert in a 2.5% agarose gel after *Bam*HI treatment of the plasmids. Clones with the correct orientation of the insert were selected by PCR, and the nucleotide sequence was confirmed by dideoxynucleotide sequencing of both strands. The BacPAK1–proHD-5$^{Met}$ transfer plasmid was purified by ultracentrifugation on cesium chloride gradients and cotransfected with *Bsu*36I-digested BacPAK6 (*Autographa californica* nuclear polyhedrosis) viral DNA into Sf21 cells with Lipofectin (Gibco-BRL, Grand Island, N.Y.). Recombinant baculovirus was harvested from culture medium 72 h after cotransfection and subjected to a plaque assay to obtain individual viral clones. Viral clones that expressed proHD-5$^{Met}$ were selected by the detection of metabolically labeled proHD-5$^{Met}$ in cell culture supernatants by taking advantage of the high cysteine content of proHD-5. [$^{35}$S]cysteine was added to Sf21 cells (100 μCi/10$^6$ cells) 1 h after infection with recombinant baculovirus. Cell culture supernatants were harvested 72 h postinfection, dialyzed against 5% acetic acid with a SpectraPor membrane with a 3.5-kDa molecular-mass cutoff, lyophilized, and subjected to acid-urea (AU)- and sodium dodecyl sulfate-Tricine-polyacryl-

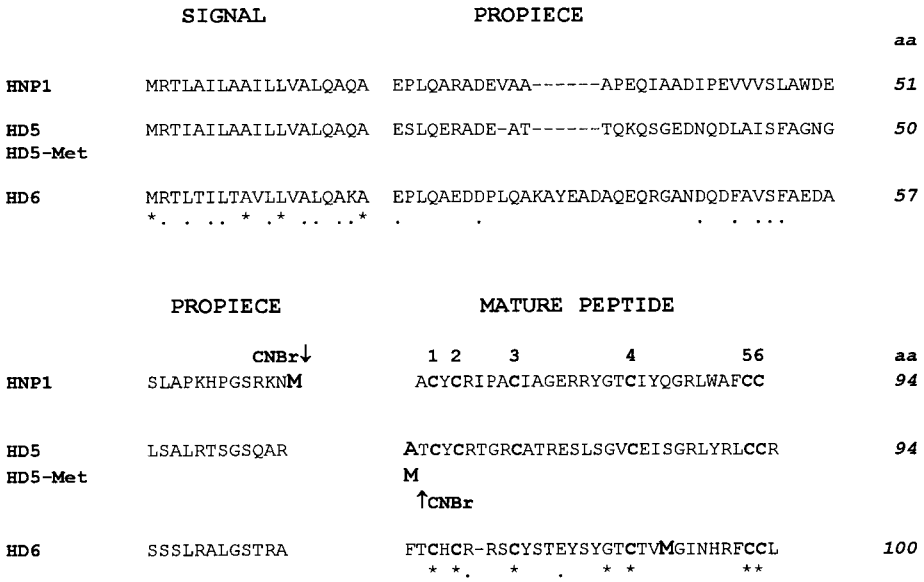


FIG. 1. Amino acid sequences of preprodefensins HNP-1, HD-5, and HD-6. Residues conserved in all known defensins are marked by asterisks, and similar residues are indicated by dots. In the mature peptide, cysteines are bolded and numbered 1 to 6. They form disulfide bonds that stabilize the peptides. Chemical CNBr cleavage of proHNP-1 was performed at the indicated native methionine residue at position 64. Cleavage of proHD-5$^{Met}$ occurred at the indicated methionine substitution for alanine at position 63. HD-6 contains a methionine residue in its mature part and is therefore unsuitable for this approach.

amide gel electrophoresis (PAGE) (38, 64). After drying, gels were exposed to XAR-5 film (Eastman Kodak, Rochester, N.Y.) at −80°C. Labeled bands on the autoradiogram of supernatants from recombinant virus were compared to those generated by wild-type virus, and the clone expressing the largest amount of $^{35}$S-proHD-5$^{Met}$ with the expected molecular mass of approximately 8 kDa was chosen for biosynthesis of rHD-5.

A baculovirus expressing unmutagenized prodefensin (proHD-5) was generated by methods similar to those described above, except that only primers A and D were used for amplification of HD-5 cDNA in a single PCR (BacPAK1–proHD-5).

**Biosynthesis and purification of rHD5.** For large-scale protein expression, *Trichoplusia ni* 5B1-4 (High Five) adherent cells (Invitrogen, San Diego, Calif.) were used, since they grow faster and yield more recombinant protein than Sf21 cells. High Five cells cultured in EX-CELL 405 serum-free medium (JRH Biosciences, Lenexa, Kans.) were seeded into 150-cm$^2$ tissue culture flasks (Corning) ($8 \times 10^6$ cells per flask), and after overnight incubation, cells at exponential growth phase (now approximately $1.5 \times 10^7$ cells per flask, which produced 60 to 70% confluency) were infected with 1 PFU of BacPAK1–proHD-5$^{Met}$ virus per cell. At 72 h after infection, cell culture supernatants were collected, centrifuged for 5 min at 800 × *g* to remove cells and cell debris, supplemented with 5 mM EDTA to inhibit proteases and with 0.1 μM β-mercaptoethanol to inhibit oxidation of the methionine residue, and stored at −20°C until processed further. A total of 1 liter of cell culture supernatants was concentrated ~10-fold and diafiltered with 5% acetic acid with a tangential flow concentration apparatus (Filtron Corp., Northborough, Mass.). Although the calculated mass of proHD-5$^{Met}$ is only 8.1 kDa, it was found to be aggregated in the culture medium and retained by a 10-kDa-molecular-mass-cutoff membrane (Filtron Omega polysulfone minisette). Concentrated proteins were lyophilized and subjected to selective chemical cleavage with CNBr based on the method described by Villa et al. (79). An equivalent of 500 ml of supernatant was dissolved in 8 ml of 6 M guanidine-HCl–0.2 M HCl, transferred into a tube containing 240 mg of CNBr, overlayed with $N_2$, and incubated overnight at room temperature in the dark. Thereafter, any remaining CNBr was hydrolyzed and removed by the addition of 32 ml of distilled water and further incubation for at least 1 h with an open cap under the fume hood. The protein mixture containing cleaved mature HD-5 (rHD-5), the propiece of proHD-5$^{Met}$, uncleaved proHD-5$^{Met}$, and other proteins of the cell culture supernatant was dialyzed against 2% acetic acid with the use of a 3.5-kDa-molecular-mass-cutoff membrane (SpectraPor; Spectrum, Houston, Tex.), lyophilized, resuspended in 4 ml of 5% acetic acid containing 3 M urea and methylene green, and separated with a continuous flow preparative gel electrophoresis apparatus (model 491 Prep Cell; Bio-Rad) in a 15.8% AU-polyacrylamide gel (27). Fractions containing rHD-5 were identified by Coomassie-stained AU-PAGE and further purified by reverse-phase high-pressure liquid chromatography (RP-HPLC) in a Vydac $C_{18}$ column (4.6 by 250 mm; Separations Group, Hesperia, Calif.) with an increment of 1% acetonitrile per min in 0.1% trifluoroacetic acid during the first 25 min followed by a 0.5% increment for 10 min and a 5% increment for the last 5 min. The concentration of rHD-5 was determined photometrically at an optical density of 280 nm with an absorption coefficient of 0.93 as calculated with PC Gene software (optical density at 280 nm [1 mg/ml] of 0.93).

**Mass spectrometry.** HPLC-purified rHD-5 was analyzed at the UCLA Center for Molecular and Medical Mass Spectrometry by laser desorption with a Voyager RP Instrument (PerSeptive Biosystems, Framingham, Mass.), and electrospray measurements were recorded on Sciex API III (Perkin-Elmer Corp., Foster City, Calif.). The mass spectrum was recorded prior to and after treatment with 6 M guanidine HCL–1 M dithiothreitol (DTT).

**Cytotoxicity.** The cytocidal activity of rHD-5 was assessed with two human intestinal cell lines by means of the colorimetric MTT [3-4, 5-dimethyl-z-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide assay (Boehringer Mannheim, Indianapolis, Ind.). The intestinal cell lines Int407 (embryonic small intestine) and Caco2 (colon adenocarcinoma) were obtained from the American Type Culture Collection (ATCC) (Rockville, Md.) and maintained at 37°C with 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM)-F12 (Gibco-BRL) with 10% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, Utah) and 50 μg of gentamicin (Gibco-BRL) per ml. Cells were detached with a trypsin-EDTA solution (0.05%, 0.53 mM; Gibco-BRL) adjusted to 500,000 cells per ml, and 50,000 cells per well were seeded onto 96-well flat-bottom tissue culture plates (Nunclon; Nunc, Naperville, Ill.). After 6 to 8 h, 11 μl of a 10-fold-concentrated rHD-5 or melittin (Sigma, St. Louis, Mo.) solution in 0.01% acetic acid was added. The addition of 0.01% acetic acid served as a solvent control, and measurements were made in triplicate. After 18 h of incubation at 37°C, the MTT assay was completed according to the manufacturer's protocol, and the absorbance reflecting the cell viability was read at 600 nm, with a reference wavelength of 650 nm. Cell viability in the presence of defensin or melittin was expressed relative to that of the solvent control.

**Coupling of rHD-5 to ovalbumin for immunizations.** Cross-linking of rHD-5 to the carrier, ovalbumin, was done with glutaraldehyde and a two-step coupling protocol for peptides with one free amino group (26). One milligram of rHD-5 in 100 μl of phosphate-buffered saline (PBS) (pH 7.4) was mixed with 100 μl of a 10% glutaraldehyde solution in PBS in a small glass vial with a magnetic microstirrer. After 30 min of stirring at room temperature, glycerol was added to a final concentration of 5%, and excess glutaraldehyde was removed by gel filtration with a 1-ml G10 Sephadex (Pharmacia) column. After the sample was loaded, the column was centrifuged for 1 min at 200 × *g*, and the glutaraldehyde-linked peptide was recovered in the first eluate and transferred to a small glass vial containing a microstirrer. Ovalbumin (Sigma) was added as the carrier protein in a 5-mg/ml stock solution in PBS (pH 7.4) until a ratio of 1 mol of peptide per 50 amino acid residues of carrier protein was achieved. After 1 h of incubation at room temperature with stirring, 1 M glycine in PBS (pH 7.2) was added to a final concentration of 200 mM, and the incubation was extended for another hour. The mixture was then dialyzed against PBS (pH 7.4) with the use of a 3.5-kDa-molecular-mass-cutoff membrane (SpectraPor), aliquoted for three separate immunizations, and stored at −20°C until use.

**Polyclonal antibodies against rHD-5.** Polyclonal antiserum was raised in female 2.5- to 3-lb New Zealand White rabbits. For the first immunization, a 200-μl

vial of ovalbumin-linked rHD-5 (containing approximately 300 μg of rHD-5) was mixed with 400 μl of physiological saline and 600 μl of complete Freund's adjuvant (Calbiochem, La Jolla, Calif.) and injected at multiple sites intradermally on the backs along the spines of two animals. Two boosts at 30-day intervals were similarly administered, except incomplete Freund's adjuvant was used instead of complete Freund's adjuvant. Prior to immunization and 10 days after each boost, blood was drawn by ear artery puncture, and the serum was stored at −20°C.

**ELISA.** The antibody titer of rabbit immune serum was determined by enzyme-linked immunosorbent assay (ELISA) as described previously (55).

**Dot blot and Western blot assays.** To test the sensitivity and cross-reactivity of the antiserum, a dot blot assay was performed. To compare the immunoreactivities of native and reduced rHD-5, Western blotting was employed. For the dot blot assay, a polyvinylidene difluoride membrane (Immobilon P; Millipore Corporation, Bedford, Mass.) was briefly soaked in methanol, followed by two 5-min washes with distilled $H_2O$. Peptides were diluted in 0.01% acetic acid and applied in 2-μl volumes onto the membrane, followed by a 30-min incubation in a humid chamber at 4°C. Unbound peptides were rinsed off with Tris-buffered saline (TBS; 500 mM NaCl, 20 mM Tris [pH 7.5]). Blocking of nonspecific antibody binding sites was done with 3% gelatin (from bovine skin, Bloom 75; Sigma) in TBS for 30 min at room temperature. Then, serial dilutions of antiserum in TBS containing 1% gelatin were added and incubated for 2 h at room temperature. The dot blot strips were washed briefly in distilled $H_2O$ and twice for 10 min each in TBS containing 0.05% Tween 20 (TTBS). Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) was diluted 1:2,000 in TBS containing 1% gelatin and added for 1 h at room temperature, followed by two washes for 10 min each in TTBS. After a brief rinse in TBS, the dot blot strips were developed with 5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium (BCIP-NBT; Sigma) as follows: to 60 ml of alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris [pH 9.8]), 10 mg of BCIP and 20 mg of NBT dissolved in 100 and 70% dimethylformamide, respectively, were added. The reaction was stopped with distilled $H_2O$. For Western blotting, 71 μM rHD-5 was incubated in 10 mM Tris–0.71 mM glycine (pH 8.0) with and without the addition of 50 mM DTT for 2 h at 37°C, subjected to AU-PAGE, and electroblotted to a polyvinylidene difluoride membrane (Trans-Blot transfer medium; Bio-Rad) for 30 min at 4°C and 0.18 mA in blotting buffer consisting of 0.7% acetic acid–10% methanol. Subsequent steps of the Western blot assay were performed as described for the dot blot assay, with a 1:2,000 dilution of polyclonal rabbit anti-rHD-5 serum.

**Immunoperoxidase staining.** Formalin-fixed tissue sections from a healthy human adult small intestine, kindly provided by W. M. Weinstein (Department of Medicine, UCLA School of Medicine, Los Angeles, Calif.) were deparaffinized by incubation in xylene twice for 3 min each, followed by two 2-min incubations with absolute ethanol, then with 90 and 75% ethanol, and finally with tap water. Thereafter, slides were washed in TBS for 5 min. In order to inactivate endogenous peroxidases, the slides were incubated in 0.1 M aqueous periodic acid for 5 min and immediately transferred to a 0.02% aqueous sodium borohydride solution for 2 min. To block nonspecific antibody binding, the slides were incubated in 3% gelatin in TBS for 1 h at room temperature prior to incubation in a 1:2,000 dilution of rabbit anti-rHD-5 serum in TBS with 1% gelatin, 0.05% Tween 20, and 0.01% merthiolate (Aldrich) for 24 h at room temperature. Incubation in a 1:2,000 dilution of rabbit preimmune serum served as a negative control. Subsequent steps of immunoperoxidase staining were performed as described by Ganz et al. (23).

**Intestinal biopsies.** Informed consents for ileal biopsies were obtained from patients undergoing routine colonoscopy for ulcerative colitis surveillance or evaluation of causes of colonic bleeding. Large-cup forceps (8-mm open span) pinch biopsies were obtained from endoscopically unremarkable mucosae at a minimum of 15 cm from the ileocecal valve. From each location, two biopsies were subjected to processing for electron microscopy. An additional biopsy from within 1 to 2 cm of the study samples was stained with hematoxylin-eosin to confirm the ileal location and the absence of microscopic inflammation.

**Immunogold stain for electron microscopy.** Biopsies of the human ileum were immediately placed for 2 h in an iced fixative solution containing 2% paraformaldehyde and 0.5% glutaraldehyde in a buffer solution of 0.08 M Na-cacodylate with 0.2% $CaCl_2$ (pH 7.3). The samples were then washed in the buffer solution for 30 min, dehydrated in graded ethanol by incubation in 50, 75, 95, and 100% ethanol for 15 min each, and embedded in LR-White (Ted Pella, Redding, Calif.). Thin sections were prepared and immunostained as previously described (23), with the following changes: blocking was performed for 30 min, and 15-nm-diameter gold bead-conjugated goat anti-rabbit IgG was employed as a second antibody. The stained samples were viewed and photographed at 80 kV on a JEOL model 100XC electron microscope.

## RESULTS

**High-yield biosynthesis of rHD-5 with the insect cell-baculovirus expression system.** Prior to large-scale production of rHD-5, the mutagenized precursor proHD-$5^{Met}$ was compared with unaltered proHD-5 to exclude major conformational changes of prodefensin due to the methionine substitution. Cell culture supernatants containing prodefensin were concentrated by ultrafiltration and purified by preparative gel electrophoresis and RP-HPLC as described for rHD-5. The electrophoretic mobilities by AU-PAGE (molecular mass as determined by sodium dodecyl sulfate-Tricine-PAGE [approximately 8 kDa]) and elution by RP-HPLC (at ~39% acetonitrile–0.1% trifluoroacetic acid) were identical for proHD-5 and proHD-$5^{Met}$.

High Five insect cells infected with BacPAK1–proHD-$5^{Met}$ (1:1 cell-to-virus ratio) released approximately 2 to 5 μg of proHD-$5^{Met}$ per ml at 72 h after infection, as estimated by Coomassie-stained AU-PAGE of cell culture supernatants. After concentration, dialysis, in vitro cleavage with CNBr, purification by preparative gel electrophoresis, and subsequent HPLC, 0.5 to 1 mg of rHD-5/500 ml of cell culture supernatant was recovered (data not shown). Its identity and purity were confirmed by mass spectrum analysis; the measured mass of the purified peptide was 3,510 Da (predicted, 3,511 Da, if all disulfide bridges are formed), and after reduction with 1 M DTT, the mass increased to 3,516 Da (predicted, 3,517 Da, for completely reduced rHD-5). These measurements indicate that the purified rHD-5 is fully disulfide cross-linked.

**Polyclonal antibodies against rHD-5 react with proHD-$5^{Met}$ and proHD-5.** Since defensins in our experience are too small to elicit a useful antiserum, rHD-5 was coupled to ovalbumin as a carrier molecule. Rabbits immunized with rHD-5–ovalbumin showed a strong immune response against uncoupled rHD-5 10 days after the second boost, with a specific antibody titer of 1:20,000 by ELISA. By dot blot analysis of the immune serum, as little as 50 ng of rHD-5 at a 1:2,000 serum dilution was detected, and the serum cross-reacted with both proHD-5 and proHD-$5^{Met}$ (data not shown).

To analyze the immunoreactivity of rHD-5 under reducing conditions, rHD-5 was treated with DTT and subjected to AU-PAGE followed by Western blotting. The polyclonal antibodies did not react with reduced rHD-5, which showed an altered electrophoretic mobility (data not shown), indicating that the antiserum is conformation specific.

**Naturally occurring HD-5 is selectively detected in Paneth cell granules by polyclonal antibodies against rHD-5.** The polyclonal sera against rHD-5 were employed in immunoperoxidase staining of normal human adult small intestine tissue sections. As shown in Fig. 2, only Paneth cells, identified by their location in the crypts and their prominent granules, stained for HD-5, but the intraepithelial neutrophils did not stain. Rabbit preimmune serum did not show any reactivity with the sections (data not shown). Thus, Paneth cells were the predominant site of HD-5 storage. To determine the subcellular location of HD-5, fresh human ileum biopsies were stained with immunogold. As shown in Fig. 3, HD-5 is concentrated in the cytoplasmic granules of Paneth cells. Since the polyclonal antibodies cross-react with proHD-5, it is not certain whether the storage form of defensin represents mature HD-5 or its precursor.

**rHD-5 exhibits little cytotoxicity to human intestinal cell lines.** Since surrounding intestinal epithelial cells would be exposed to HD-5 upon Paneth cell degranulation, the cytotoxic activity of recombinant HD-5 was assessed with two intestinal cell lines. rHD-5 did not exhibit cytotoxic activity against Caco2 as determined by the metabolic MTT assay; even in the presence of 100 μg of rHD-5 per ml, more than 90% of the cells remained viable (Fig. 4A). Against Int407, rHD-5 at 100 μg/ml showed some cytotoxic activity, with reduction of cell viability to 67% ± 14% (mean ± standard deviation) (Fig. 4B). In contrast, 25 μg of melittin per ml reduced cell viability to 69% ± 16% and 4% ± 1.5% for Caco2 and Int407, respec-

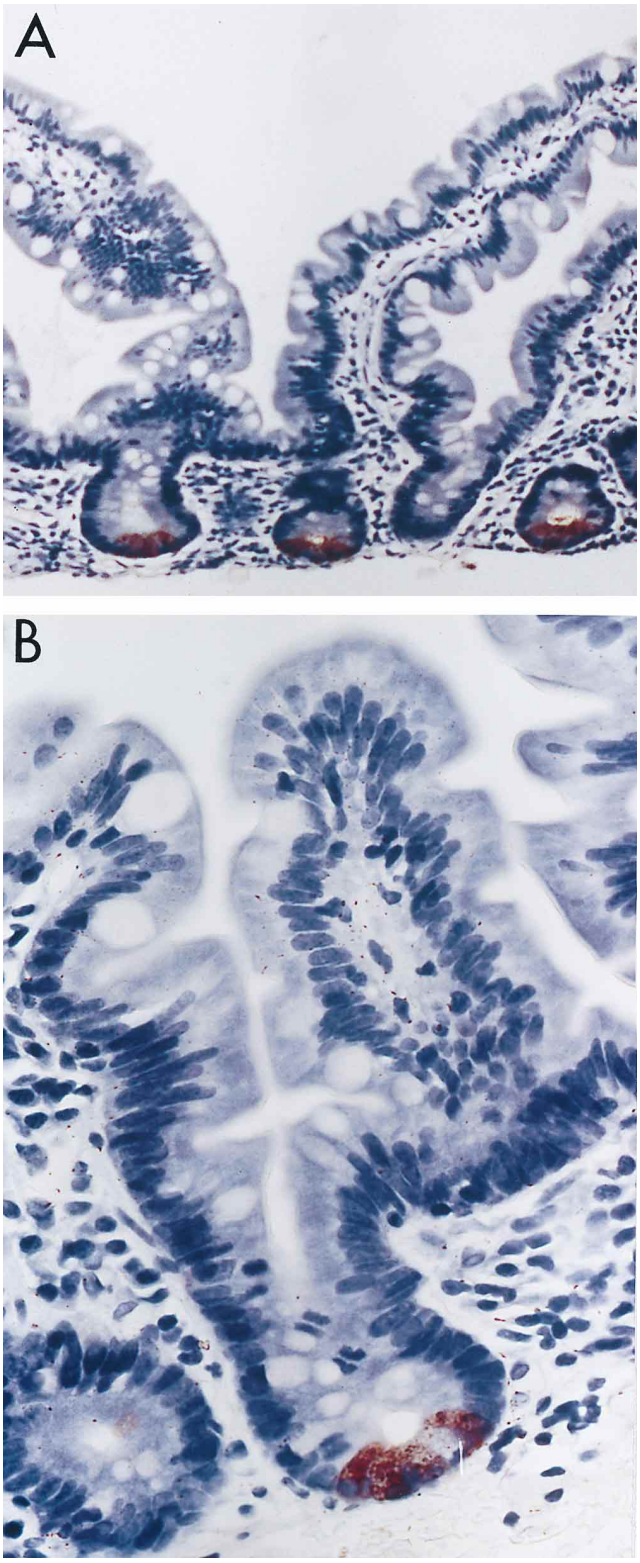


FIG. 2. Immunoperoxidase staining of Formalin-fixed normal human small intestine biopsies with polyclonal rabbit antibodies against rHD-5. Slides were counterstained with hematoxylin. Only Paneth cells located in the crypts contained the brown peroxidase product. Magnification, 200-fold (A) and 500-fold (B).

tively. proHD-5$^{Met}$ or solvent was not cytotoxic to either cell line (data not shown).

## DISCUSSION

We biosynthesized HD-5 in high yield in the baculovirus-insect cell expression system, which had also been successfully employed for the production of HNP-1 (78). Insect cells infected with the HD-5–baculovirus construct released prodefensin into the culture medium. To obtain mature HD-5 we generated a methionine substitution in proHD-5 for selective chemical cleavage with CNBr (59). Since the amino terminus of naturally occurring HD-5 forms has not been identified yet, we selected a likely processing site based on the sequence of HNP-1.

Most of the purified mouse intestinal defensins (cryptdins) share an N-terminal sequence somewhat longer than the sequences of many myeloid defensins of other species and contain five amino acids N-terminal of the first conserved cysteine (16, 28, 69). However, mice lack myeloid defensins for comparison to the intestinal defensins (17). In cattle, some myeloid β-defensins contain four amino acid residues N-terminal of the first conserved cysteine (70), as does the epithelial tracheal β-defensin (15). In addition, the lengths and the sequences of the N-terminal amino acid residues of purified cryptdins differed depending on the mouse strain (28, 69), and cryptdins also undergo extracellular proteolytic processing that shortens their N termini (68a). Notably, cationic and anionic trypsins were found in Paneth cells (8, 71) and could cleave at the arginine residue located at our proposed cleavage site. Furthermore, enterocytes contain membrane-bound aminopeptidase N, which can cleave any N-terminal amino acid residue (52, 75). Therefore, even if naturally occurring HD-5 is first released with an N terminus longer than that which we proposed, multiple shorter forms are likely to be generated in the intestinal lumen.

To generate the CNBr cleavage site, we performed a methionine substitution in proHD-5. The proregion of HNP-1 is essential in subcellular trafficking (41) and for neutralizing the activity of mature HNP-1 (78). As has been shown for other proteins, exchange of a single amino acid might alter protein conformation (72), stability (11), and function (12, 72, 80). Thus we considered the possibility that recombinant proHD-5$^{Met}$ would yield a conformationally altered mature peptide. However, the intact proHD-5 and proHD-5$^{Met}$ behaved identically by AU-PAGE as well as RP-HPLC, precluding major conformational alterations due to the methionine substitution. Moreover, rHD-5 and naturally occurring HD-5 must also have similar conformations since the antibodies generated against fully disulfide-bonded rHD-5 reacted with naturally occurring intestinal defensin in tissue at high dilutions of antiserum but failed to recognize the conformationally altered reduced form of rHD-5.

In this first report of subcellular localization of an intestinal defensin peptide, HD-5 was detected by immunoperoxidase staining and immunogold staining exclusively in Paneth cell granules. Since our polyclonal antibodies against rHD-5 cross-react with its precursor proHD-5, we cannot determine whether intestinal defensin is stored in the granules in its mature form or as a propeptide. Although known myeloid defensins are stored in granules in their mature form, bactenecins, bovine neutrophil antimicrobial peptides, are stored in the large granules as probactenecins and cleaved to their active mature forms by neutrophil elastase only after degranulation (68). Paneth cell trypsin, intraluminal digestive proteases, and enterocyte aminopeptidase N could also process prodefensins

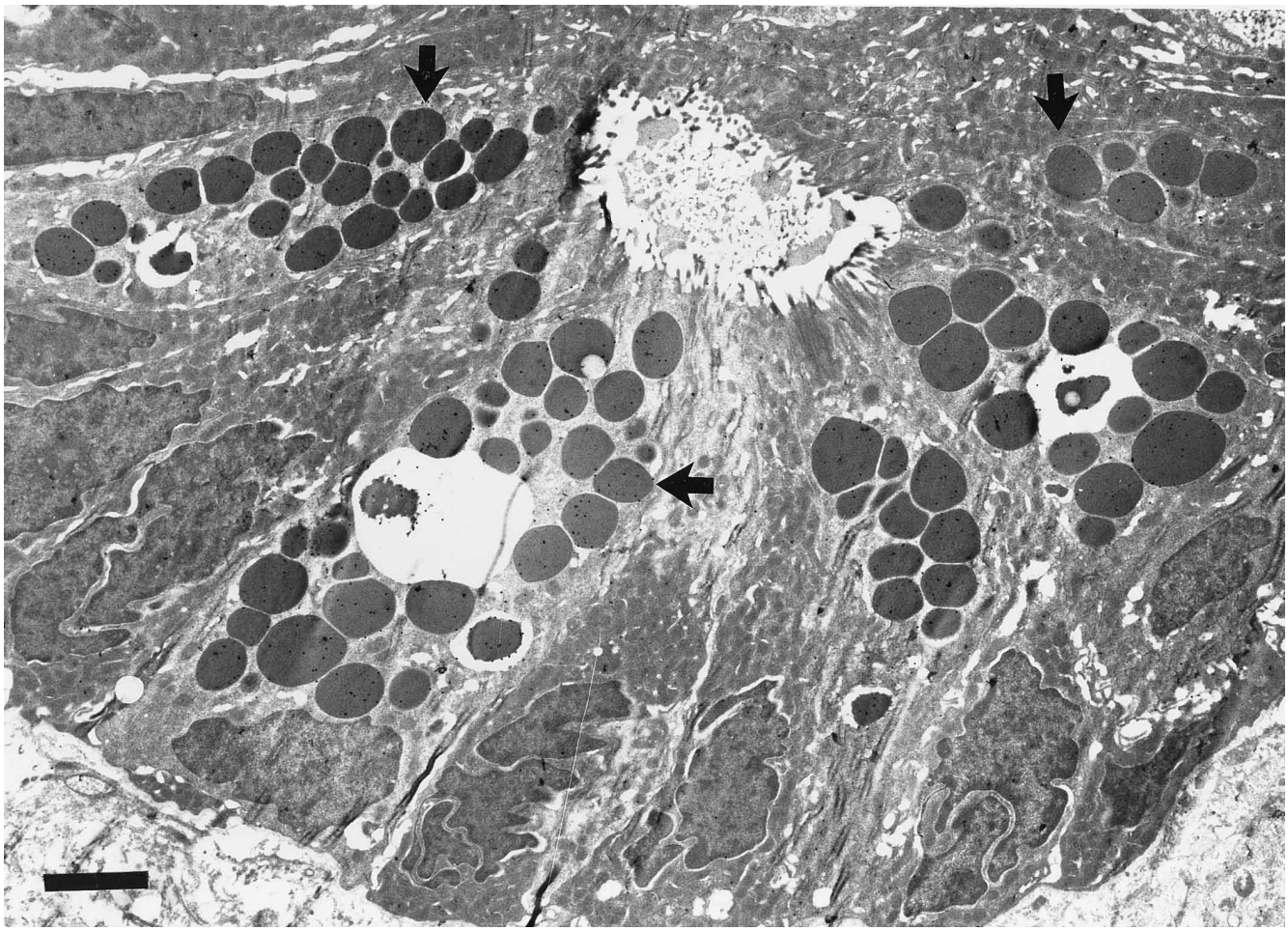

FIG. 3. Transmission electron micrograph of human ileum crypt with immunogold staining for HD-5. Defensin-rich granules (arrows) were found exclusively in Paneth cells. Bar, 2 μm.

to mature defensins or merely trim released mature defensins to shorter forms.

Selective staining of Paneth cells with the polyclonal rabbit antibodies against HD-5 is in agreement with the localization of mRNA for HD-5 and HD-6 in the same cell type (31, 32). Unlike in situ hybridization, immunostaining can detect defensin in cells that do not actively synthesize the peptide but either accumulate it during prior differentiation stages (14) or acquire it from other cell types. We saw no evidence of such cells in the immunostained sections of the small bowel. The specificities of the antibodies qualify them as a Paneth cell marker that could enhance Paneth cell identification in intestinal metaplasia (36, 53), in tumors with enteric differentiation (1, 46), and in inflammatory bowel disease such as chronic diverticular disease (42), microscopic colitis (19), Crohn's disease (65), ulcerative colitis (50), and pouchitis (3). Previously used Paneth cell markers such as lysozyme (25, 58, 60), phospholipase $A_2$ (34, 49), alpha 1-antitrypsin (47), pancreatic secretory trypsin inhibitor (9, 20), pokeweed lectin (18), and CD15 (4) are not exclusive to Paneth cells but stain other gastrointestinal epithelial cells, intraepithelial lymphocytes, or myeloid cells. Furthermore, the presence of intestinal defensin in Paneth cells could also be used as a developmental marker of Paneth cell maturation (10, 43).

Upon stimulation of Paneth cells, their granules are released into the intestinal lumen (45, 61, 62), and intestinal defensin (mature HD-5 or readily cleaved proHD-5) is likely to be delivered in close proximity to neighboring epithelial cells. Since some defensins exhibit cytotoxic activity at high concentrations (40, 51) and promote growth of epithelial cells and fibroblasts (48), we assayed the cytotoxic and mitogenic activities of rHD-5 against human intestinal cell lines. rHD-5 (and proHD-$5^{Met}$) did not exhibit any mitogenic activity. It was not cytotoxic to the more differentiated cell line Caco2 (21, 73), and only minor cytotoxic activity was observed at high concentrations against the much less differentiated cell line Int407 (21, 73). Since melittin, a very potent pore-forming toxin (7), also had more effect on Int407 than on Caco2, the latter, more differentiated cell line may utilize a specific repair mechanism or may be intrinsically resistant to membrane pertubation (33, 81).

The location of intestinal defensin in Paneth cell granules, previously reported to also contain lysozyme (45, 58), phospholipase $A_2$ (34, 49), TNF-α (5, 66), and secretory IgA (63), implicates these cells in mucosal defense against microorganisms. Antimicrobial activity was demonstrated for murine cryptdins (2, 16, 28, 69), and in a parallel publication we describe the antimicrobial properties of rHD-5 (55a). The antimicrobial effects of defensins and other microbicidal substances from Paneth cells are likely to be exerted near the site of their secretion, before dilution and potential inhibitors diminish their activity. Since Paneth cells are located adjacent to

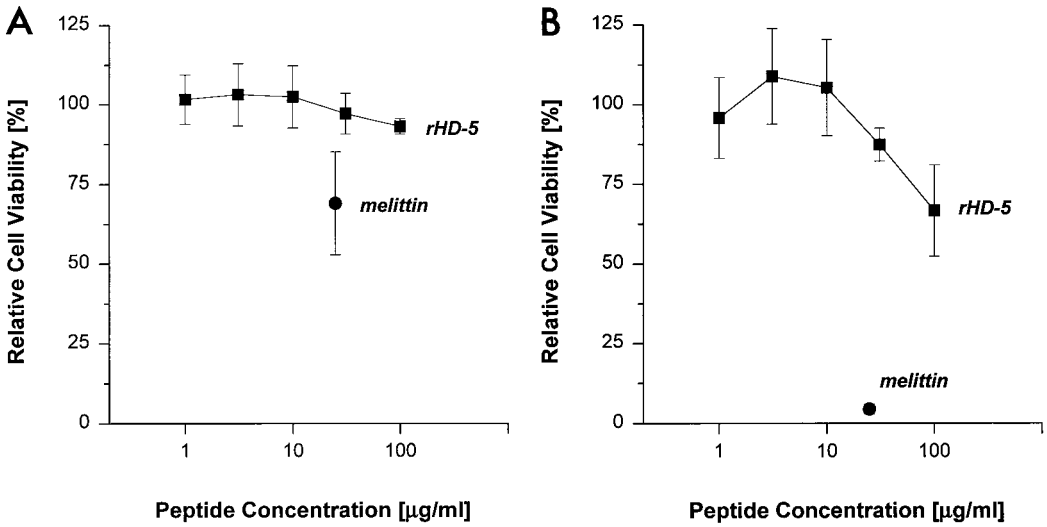


FIG. 4. Cytotoxic activities of rHD-5 against human intestinal cell lines determined by colorimetric metabolic assay. Absorbance was measured after 18 h of incubation with or without rHD-5, and values obtained for control cells were set as 100% viability. Bee melittin was employed as a reference for cytotoxic action. Depicted data points are means ± standard deviations for three experiments performed in triplicate. (A) Caco2, colon adenocarcinoma; (B) Int407, embryonic small intestine.

the mitotic cells that generate and regenerate the intestinal epithelium, they may function to protect these critically important cells against invasion by microbes.

## ACKNOWLEDGMENTS

We thank Erika V. Valore for aid in the biosynthesis of rHD-5; Sylvia S. Harwig, Leonie Tan, and Hye Jin Yang for their contributions to the purification of proHD-5, proHD-5$^{Met}$, and rHD-5; and Alexander Panyutich for assistance in the generation of polyclonal antibodies against human intestinal defensin. We acknowledge the technical assistance of Kym Faull at the UCLA Mass Spectrometry Facility.

This work was supported in part by Public Health Service-National Institutes of Health grants HL-35640 and HL-46809.

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*Editor:* J. R. McGhee