Human β-Defensin-1: An Antimicrobial Peptide of Urogenital Tissues

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Abstract

Antimicrobial peptides are widely distributed mediators of innate host defense in animals and plants. A 36 amino acid antimicrobial peptide belonging to the defensin family, and named human β-defensin-1 (HBD-1), was purified recently from hemodialysate fluid, but its tissue sources were not identified. By Northern blotting, we found the highest concentrations of HBD-1 mRNA in the kidney and the female reproductive tract. In situ hybridization localized the HBD-1 mRNA in the epithelial layers of the loops of Henle, distal tubules, and the collecting ducts of the kidney and the epithelial layers of the vagina, ectocervix, endocervix, uterus, and fallopian tubes in the female reproductive tract. Using a novel technique designed to detect cationic peptides in urine, we recovered several forms of HBD-1 ranging in length from 36 to 47 amino acid (aa) residues and differing from each other by amino terminal truncation. The total concentration of HBD-1 forms in voided urine was estimated at 10–100 μg/liter, with individual variations in the total amount of HBD-1 peptides and the relative proportion of HBD-1 forms. Multiple forms of HBD-1 (size 36–47 aa) were also found in the blood plasma, bound to carrier macromolecules that released the peptide under acid conditions, and in vaginal mucosal secretions (39, 40, and 44 aa). By immunostaining, HBD-1 was located in the kidney within the lumen of the loops of Henle, but no intracellular storage sites were identified in renal or female reproductive tissues. Recombinant HBD-1 forms (36, 39, and 42 aa) and natural HBD-1 forms were antimicrobial to laboratory and clinical strains of *Escherichia coli* at micromolar concentrations. HBD-1 activity was not changed appreciably by low pH, but was inhibited by high salt conditions. Some of the HBD-1 peptides retained their activity against *E. coli* in unconcentrated (low conductance) urine, and the 36 aa form was microbicidal even in normal (high conductance) urine. Production of HBD-1 in the urogenital tract could contribute to local antimicrobial defense. (*J. Clin. Invest.* 1998. 101:1633–1642.)

Key words: antibiotics, peptide—chemistry • antiinfective agents—analysis • epithelium—chemistry • genitalia, female—chemistry • peptide—purification

Introduction

The defensins are a family of six cysteine 3–4-kD cationic peptides with a characteristic fold that is common to the two subfamilies, α- and β-defensins, despite a difference in the connectivity of their disulfide bonds. Most defensins are antimicrobial peptides that are thought to act by selective disruption of the target membranes. Defensins are particularly abundant in the microbicidal granules of polymorphonuclear leukocytes (1), but more recently epithelial defensins have been identified in rodent and human intestinal Paneth cells (2–8) and in bovine tracheal epithelium (9, 10). The production of some epithelial defensins is augmented by infection or injury (11–15).

A novel human β-defensin (HBD-1)[1] was purified from human blood, its amino acid sequence was determined, and a cDNA fragment corresponding to its mature sequence was cloned from kidney and vaginal cDNA libraries by PCR with degenerate primers (16). We cloned the HBD-1 gene and located it within 150 kb of the α-defensin cluster on chromosome 8, band p23 (17), thus showing that HBD-1 and the previously characterized neutrophil and Paneth cell defensins evolved from a common ancestral gene. As assayed by reverse transcriptase–PCR, HBD-1 mRNA was found to be widely distributed in epithelial organs (18). Human respiratory epithelia maintained in nude mice generated fluid with antimicrobial activity that was abolished by treatment of the epithelia with antisense oligonucleotide to HBD-1 (19), indicating that the peptide is likely to play an important role in epithelial host defense. However, the characteristics of the peptide itself remained largely unknown. The present study explored the production, distribution, and activity of HBD-1 peptide in human tissues.

Methods

Human tissue. Under protocols approved by the Institutional Review Boards, anonymous discard tissues from the female reproductive tract from operations for nonmalignant disorders were placed on ice, and in some cases dissected into mucosa-enriched and stromal layers. Within a few hours after surgery, the tissue fragments were minced with fine scissors and frozen in liquid nitrogen or fixed in 10% neutral formalin. Discard renal specimens preserved in liquid nitrogen or as neutral formalin-fixed paraffin-embedded sections were ob-

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1. *Abbreviations used in this paper:* aa, amino acid; AU-PAGE, acid-urea PAGE; CFU, colony forming units; CM, carboxy methyl; HBD-1, human β-defensin-1; RP-HPLC, reverse phase HPLC; TBS, Tris buffered saline; TTBS, Tris buffered saline with 0.05% Tween 20.

tained from the UCLA Human Tissue Research Center or from Dr. Cynthia Nast (Department of Pathology, UCLA). In some cases, renal tissue was dissected into cortex, superficial medulla, and deep medulla before storage in liquid nitrogen.

Expression of human β-defensin-1 mRNA. The frozen tissue mince was lysed at room temperature by vigorous vortexing in a mixture of 4 M guanidine isothiocyanate solution and RNase-free sand (20). Total RNA was extracted with phenol-chloroform and selectively precipitated in isopropanol as previously described (21). When larger amounts of tissue (0.1–0.5 g) were available, tissue was snap-frozen and ground to a powder with a mortar and pestle; then RNA was extracted and purified by the guanidinium isothiocyanate–cesium chloride centrifugation method (22), or using Trizol reagent (Gibco Laboratories, Grand Island, NY) according to the manufacturer's instructions. RNA bands were separated on 1% agarose formaldehyde gels, stained with acridine orange dye, and transferred to Gene Screen Plus (Dupont-NEN, Boston, MA) nylon membranes (23). Equal transfer and RNA integrity was confirmed by photographing the acridine-stained RNA on membranes under ultraviolet illumination and, when necessary, by hybridization with a human actin probe (Clontech, Palo Alto, CA). Two commercial preloaded membranes with human mRNA from spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood leukocyte, heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas were obtained from Clontech. The HBD-1 cDNA was labeled by the random oligonucleotide method and hybridized to the immobilized RNA for 18 h in 50% formamide, 0.75 M sodium chloride, 0.05 M Tris-HCl, pH 7.5, 1% SDS, 0.5% powdered milk, and 10% dextran sulfate at 42°C. Blots were washed at high stringency (1.5 mM sodium citrate, pH 7, containing 15 mM NaCl, at room temperature) and exposed to Biomax MS film with Biomax intensifying screen (Eastman Kodak Co., Rochester, NY). For some Northern blots, the HBD-1 mRNA levels were normalized to actin mRNA by scanning the intensity of each band on the autoradiogram using a laser densitometer (Molecular Dynamics, Sunnyvale, CA).

In situ hybridization. Paraffin-embedded tissue samples were prepared for use with in situ hybridization as previously reported (24). Tissues processed within 1.5 h of excision were fixed at room temperature by overnight immersion in 10% ultrapure EM grade formaldehyde (Polysciences, Inc., Warrington, PA) embedded in paraffin, sectioned to 5-mm thickness and mounted onto glass slides (Superfrost Plus; Fisher Scientific Co., Fairlawn, NJ), which were air dried and stored at room temperature until they were used for hybridization.

The cDNA used for in situ hybridization consisted of the entire HBD-1 open reading frame (antisense, 317 nucleotides; sense, 312 nucleotides) cloned into the pCR 3.1 TA vector (Invitrogen Corp., Carlsbad, CA) as previously described (25). Purified plasmid was linearized with the appropriate restriction enzyme, and sense or antisense 35S-UTP, 35S-CTP (Amersham Corp., Arlington Heights, IL) double-labeled probes prepared using the Riboprobe Gemini II Core System transcription kit (Promega Corp., Madison, WI).

Hybridization was performed on triplicate sections from the same tissue for each of three exposure times (2, 4, and 5 wk). Hybridization steps were conducted according to standard methods (26) as modified by Matsushita et al. (24). Briefly, just before hybridization, the slides were rinsed with 2× SSC (300 mM NaCl, 30 mM sodium citrate), and then treated with 1 μg/ml proteinase K in 100 mM Tris, 50 mM EDTA, pH 8, at 37°C for 40 min, rinsed in PBS and acetylated with 0.1 M triethanolamine and 0.25% acetic anhydride at room temperature for 10 min. The slides were then rinsed with 2× SSC, dehydrated in graded ethanols, and air dried. Riboprobe was denatured at 80°C for 3 min before adding to the hybridization mix containing 1× Denhardt's solution, 50% deionized formamide, 0.3 M NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 0.5 mg/ml yeast tRNA, 80 μg/ml denatured salmon sperm DNA, 10 mM sodium phosphate, 10% dextran sulfate, and 0.1 M dithiothreitol. Probe was added to a concentration of ~ 12,000 cpm/μl, and 50 μl of the hybridization solution was spread over the section and allowed to hybridize at 50°C for 16–20 h in a humidified chamber. After hybridization, sections were washed at high stringency (50% formamide, 2× SSC, and 25 mM dithiothreitol) at 60°C for 30 min, rinsed with 4 M NaCl, 10 mM Tris, pH 8.0, 5 mM EDTA, and 25 mM dithiothreitol, and treated with 20 mg/ml RNase A and 1 U/ml RNase T1 for 30 min at 37°C. After a second series of high stringency washes and dehydration, the sections were coated with NTB-2 autoradiography emulsion (Eastman Kodak Co.), diluted 1:1 in distilled H_2O, air dried, and exposed at 4°C before being developed, and then counterstained with toluidine blue.

Production of recombinant human β-defensin. Recombinant HBD-1 peptides were produced using an insect cell/baculovirus protein expression system as described previously for other defensins (8, 27). The cloning of the HBD-1 cDNA and gene is described elsewhere (17). To make a baculovirus gene construct, the HBD-1 cDNA was modified for insertion into the BacPAK9 transfer vector (Clontech) by PCR with oligonucleotides that incorporated BamHI and EcoR1 enzyme restriction sites into the ends (sense oligomer A: 5′-CTGGGATCCTGAGTGTTGCCTTGCCAGTC-3′ and antisense oligomer D: 5′-AAGGAATTCTCACTTCTGCGTCATTTCTTC-3′). For the production of the 36 amino acid form of HBD-1 detected in blood (16), the serine in position 32 of the HBD-1 prepropeptide was mutated to methionine for cyanogen bromide cleavage as described previously (8). Briefly, two intermediate fragments were generated. The first one was made with the sense oligomer A and an antisense oligomer B (5′-GCTGACGCAATTGTAATGATCCATTCTGTGGC-3′) that altered the serine codon to a methionine codon. The second fragment was generated with the antisense oligomer D and a sense oligomer C (5′-GATCATTACAATTGCGTCAGC-3′) that was designed to overlap the mutant (methionine) fragment. The final PCR product was generated by using the two fragments (A–B and C–D) as a template in an overlap extension reaction (28) with the primers A and D. The HBD-1 cDNA inserts were ligated into the BacPak 9 transfer vector after restriction digest with BamHI and EcoR1, and then transformed and amplified in *Escherichia coli* XL-1 blue. HBD-1-containing clones were selected and the HBD-1 sequence verified by dideoxynucleotide sequencing on both strands.

After cotransfection of the transfer vector and a defective baculovirus into Sf21 insect cells, viable recombinant Baculoviral clones were selected that produced a cationic protein on acid urea PAGE (AU-PAGE) not seen with control baculovirus. The identity of the peptide was confirmed by amino acid sequencing. The baculovirus was then used to infect insect cells at a concentration of 5 plaque forming units/cell. Recombinant peptides were purified from the culture media after 96 h of infection. The cationic recombinant peptides were adsorbed to a carboxy methyl (CM) Macro-Prep ion exchange column (Bio-Rad Laboratories, Hercules, CA) using 20 ml of a 50% slurry equilibrated in phosphate buffered saline, eluted with a gradient from 25 mM ammonium acetate, pH 7.5, to 5% acetic acid, and further purified by reverse phase HPLC (RP-HPLC) as described below for urinary peptides. For production of the 36 amino acid (aa) HBD-1 peptide, the HBD-1_{met} peptide was subjected to CNBr (cyanogen bromide) cleavage after the initial purification by CM Macro-Prep beads. Samples were treated with 1 M CNBr in 70% trifluoroacetic acid (TFA) at a peptide concentration of 1 mg/ml, overlaid with N_2 gas, and incubated at room temperature overnight in the dark. Excess CNBr was hydrolyzed with water and TFA was removed by lyophilization of the sample. Cleavage products were separated by continuous flow AU-PAGE (29), followed by RP-HPLC as described below.

Purification of cationic peptides from urine. Human urine was collected over several days in 1–10-liter containers and stored at 4°C. Cells and other particulates were removed by centrifugation at 1,000 *g* or filtration with filter paper (No. 4; Whatman Inc., Clifton, NJ). Cationic substances were extracted from urine by mixing with weak cation exchange beads: 1 ml of 50% slurry/liter of urine (CM Macro-Prep). Large volumes were extracted by stirring the mixture for 16 h at 4°C, and then the beads were sedimented at 200 *g*, poured into a column, and washed with 25 mM ammonium acetate, pH 7.5, until a

stable absorbance at 280 nm was achieved. The adsorbed cationic substances were released from the column by elution with a linear gradient from 25 mM ammonium acetate, pH 7.5, to 5% acetic acid. Small volumes (1–200 ml) were extracted by mixing for 1–2 h at room temperature. Peptides bound to CM Macro-Prep beads were washed five times with approximately two bed volumes of 25 mM ammonium acetate, pH 7.5, and then batch extracted by adding two volumes of 5% acetic acid and incubating for at least 20 min at 4°C. Fractions that migrated similarly to defensin peptides on Coomassie-stained acid-urea PAGE were pooled and further purified by RP-HPLC on a 4.6 × 250 mm Vydac C18 column (Separations Group, Hesperia, CA) using a 1% acetonitrile increment per minute in 0.1% TFA during the first 20 min, followed by a 0.5% increment for 20 min and a 1% increment for the last 30 min (30).

Purification of cationic peptides from tissues. Frozen kidney was pulverized using a mortar and pestle in liquid nitrogen and dissolved in Trizol reagent (~ 150 mg/ml) and proteins purified according to the manufacturer's protocol. Protein pellets were then extracted with 5% acetic acid overnight. Alternatively, pulverized tissues were extracted with 5% acetic acid and after sonication to disrupt the cells, incubated overnight at 4°C with rotation. Acid-soluble proteins were lyophilized, resuspended in a minimal volume of 0.01% acetic acid (~ 500 μl), diluted 20-fold with water and neutralized with NaOH to pH 7.0–7.5. CM Macro-Prep was used to extract cationic peptides for 2 h with rotation at room temperature; then the beads were allowed to settle overnight at 4°C. After washing the beads with 25 mM ammonium acetate, pH 7.5, proteins were batch eluted with 5% acetic acid.

Purification of cationic peptides from blood. Approximately 50 ml blood was drawn from male and female human subjects into tubes containing 2 mM EDTA as an anticoagulant. The blood was centrifuged at 1,500 *g* to sediment cells. The remaining blood plasma was filtered through a 10-kD molecular weight cut off centrifuge membrane (Omega Macro-Prep filter; Pall-Filtron, Northborough, MA) at 5,000 *g* for 2-h intervals. The retentate was washed with water until two sample volumes of filtrate were collected. To dissociate acid-soluble proteins from larger complexes, the retentate was acidified with concentrated acetic acid to 5% final concentration and washed with 5% acetic acid until two to three sample volumes were collected. The acid wash was lyophilized, and then resuspended in water. Both the water and acid washes were neutralized to pH 7.5 and extracted with 100 μl CM Macro-Prep (50%) slurry for washes from each 10 ml blood plasma. Cationic proteins were batch eluted from the beads with 5% acetic acid after extensive washes with 25 mM ammonium acetate, pH 7.5.

Purification of cationic peptides from vaginal lavage. Vaginal lavage fluid was obtained from a healthy 37-yr-old mid-cycle woman after douching with approximately 200 ml of a commercially available vinegar and water douche (Massengill, extra cleansing douche; SmithKline-Beecham, Pittsburgh, PA). After cells were removed by centrifugation, the lavage fluid was neutralized to pH 7.0 with ammonium hydroxide and extracted with 1 ml 50% CM Macro-Prep slurry by mixing for 2–4 h at room temperature, and then the beads were allowed to settle overnight at 4°C. After washing the beads with 25 mM ammonium acetate, pH 7.5, proteins were batch eluted with 5% acetic acid and further purified by RP-HPLC.

Identification of peptides. The major HPLC peptide peaks were individually collected and analyzed by AU-PAGE. For Western blot, peptides were transferred from acid-urea gels to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA) for 40 min with 0.7% acetic acid and 10% methanol at 0.18 amperes using a Transblot apparatus (Bio-Rad Laboratories). Western blots on nitrocellulose were found to be 10-fold less sensitive than those on PVDF membranes. Blots were fixed for 4 h with 10% formalin in Tris-buffered saline (TBS), blocked for 30 min in 3% gelatin in TBS, and then incubated for 18 h in 1:1,000 rabbit anti–HBD-1 serum (described below) diluted in 1% gelatin-TBS solution containing 0.01% thimerosal as a preservative. The blots were washed in TBS with 0.05% Tween 20 (TTBS) three times for 10 min each, and then incubated in a 1:2,000 dilution of alkaline phosphatase (AP)–conjugated goat anti–rabbit IgG in 1% gelatin-TBS for 1 h, and then washed with TTBS and developed in AP development solution (31) (bromochloroindolyl phosphate/nitroblue tetrazolium). Peptides yielding single Coomassie-stained bands and positive reaction on Western blot were analyzed in the UCLA Center for Molecular and Medical Mass Spectrometry by laser desorption mass spectrometry on the Voyager RP Instrument (PerSeptive Biosystems, Framingham, MA) and electrospray ionization mass spectrometry on Sciex API III (Perkin-Elmer Corp., Foster City, CA). NH_2-terminal amino acid sequencing was performed at the UCLA Peptide Sequencing Facility.

Immunolocalization of human β-defensin-1 peptide. Rabbit polyclonal antiserum to HBD-1 was produced by immunization with the 39 aa rHBD-1. Antigen was coupled to ovalbumin with glutaraldehyde using a single step cross-linking technique (31), and rabbits were immunized by intradermal injection. For immunostaining, deparaffinized sections from tissues fixed with 10% formalin in PBS were treated to inactivate endogenous peroxidase by incubation for 5 min in 0.1 M aqueous periodic acid, and then 2 min in 0.02% aqueous sodium borohydride and washed in Tris-buffered saline (TBS: 500 mM NaCl, 20 mM Tris, pH 7.5). Slides were subsequently incubated with 1:500 dilution of rabbit polyclonal serum in 1% gelatin (Bovine skin 75 bloom; Sigma Chemical Co., St. Louis, MO), TBS, 0.05% Tween 20 (Sigma Chemical Co.), and 0.01% thimerosal for 18 h at room temperature. The first antibody solution also contained 1:1,000 dilution of nonspecific goat antiserum to aid in blocking nonspecific binding sites. After three 10-min washes in TTBS, the slides were incubated with horseradish peroxidase–conjugated goat anti–rabbit IgG (Bio-Rad Laboratories) diluted 1:2,000 in 1% gelatin solution for an additional 18 h, washed in TTBS, and developed for 1–3 min in diaminobenzidine solution (30 mg diaminobenzidine [Bio-Rad Laboratories] dissolved in 50 ml of 50 mM Tris, pH 7.6, and filtered through a No. 4 filter, and 50 μl 30% hydrogen peroxide added just before use). Slides were counterstained with Harris Hematoxylin stain (Fisher Scientific). Controls included preimmune serum (drawn before immunization) and HBD-1-extracted immune serum. To remove HBD-1-specific antibodies, rabbit anti–HBD-1 serum was first extracted twice on an affinity column prepared by binding HBD-1 (39 aa) to the column using an Aminolink immobilization kit (Pierce, Rockford, IL). To further extract the HBD-1-specific antibody, the antiserum was incubated for 2 h with strips of nitrocellulose and Immobilon-P PVDF membrane that contained 10–13 dots of 0.6 μg HBD-1 (44 aa form) in each dot, fixed for 4 h with 10% formalin.

Antimicrobial assays. The colony forming units (CFU) assay was performed as previously described (8) using the laboratory strain *E. coli* ML-35p and a fresh isolate (No. 82716) from a patient with a urinary tract infection that was sensitive to antibiotics. Two urine samples from the same individual were used for all of the assays; low salt urine, pH 7.1 (urine collected after drinking a high volume of water), had a conductance of 1.09 mS and high salt (normal) urine, pH 6.5, which had a conductance of 9.22 mS. For comparison, the assay was also done using 10 mM $NaPO_4$ buffer, pH 7.4 (conductance = 1.58 mS). Briefly, *E. coli* and HBD-1 peptide were mixed at 37°C for 3 h in the presence of sterile filtered 10 mM PO_4, pH 7.4, low salt urine, or high salt urine. After the allotted incubation time, the reaction was stopped by 1:100 dilution in ice cold 150 mM NaCl, 10 mM PO_4, pH 7.4. Bacteria were spread on agar plates with a spiral plater (Spiral Biotech, Inc., Bethesda, MD) that delivers a known volume per area and thus allows precise counts of the bacterial colonies.

Cleavage of HBD-1 peptide with AspN endoproteinase. Peptide was lyophilized and resuspended in 2 μl of 0.01% acetic acid. AspN endoproteinase (Sigma Chemical Co.) dissolved in 10 mM Tris, pH 8.0, was added so that an enzyme to peptide ratio of 1:100 was achieved. The mixture, in a final volume of 50 μl, was incubated at 37°C for 2–3 h and the reaction stopped by adding 200 μl of 5% acetic acid. Cleaved peptides were further purified by RP-HPLC (described above).

Results

Tissue expression of human β-defensin. The mature HBD-1 peptide was originally isolated from human blood filtrate, and the corresponding cDNA fragment was cloned from kidney and vaginal cDNA libraries (16). To identify the major sites of HBD-1 expression, we performed Northern blot analysis of two standardized organ panels of human mRNA (Clontech) with our full length HBD-1 cDNA probe. In agreement with a previous report (18), the most intense hybridization signal was seen in the pancreas and kidney RNAs, with trace hybridization in the liver (not shown). A third mRNA panel containing bone marrow mRNA and mRNA from lymphoid organs (Clontech, not shown) showed no hybridization, suggesting that the HBD-1 mRNA detected in the kidney did not originate from myeloid or lymphoid cells. Northern blots from various regions of the female reproductive tract (Fig. 1) documented HBD-1 mRNA expression predominantly in the endocervix, ectocervix, and the vagina. Additional Northern blots (data not shown) containing RNA from epithelium-enriched samples detected HBD-1 mRNA in the vagina, ectocervix, endocervix, endometrium, and fallopian tubes, but not in uterine stroma/myometrium. Among these samples, hybridization to endocervical RNA yielded the strongest and most consistent signal in multiple specimens, whereas the endometrium exhibited the most variable levels of HBD-1 mRNA expression. In the kidney, in situ hybridization (Fig. 2) of antisense probes localized the HBD-1 mRNA in the columnar epithelial layers of the distal tubules in the renal cortex (*RC*), the loops of Henle, and the collecting ducts of the renal medulla (*RM*). In the female reproductive tract, HBD-1 mRNA was localized to the columnar epithelial layers of the uterus (Fig. 2, *U*), endocervix (*EC*), and ectocervix (not shown), with low level hybridization also seen in the fallopian tubes (*FT*). HBD-1 mRNA was also detected in the basal layers of the squamous epithelium of the vagina (*V*). As was expected, hybridization of sense probes yielded only low level background signal in all tissue studied.

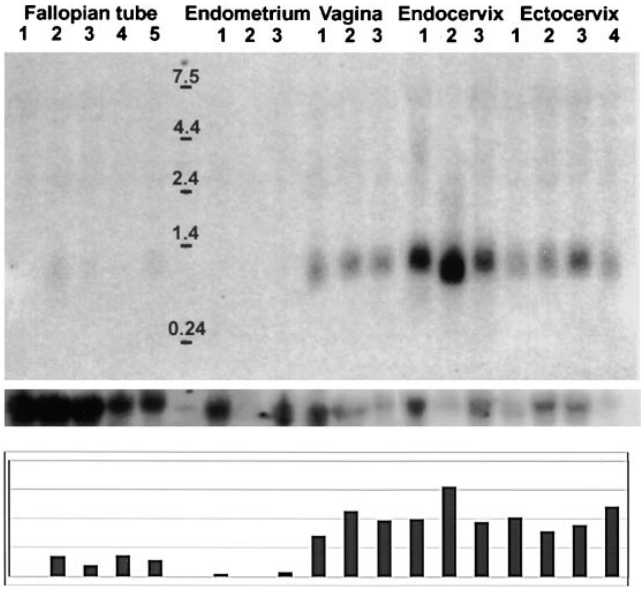

Figure 1. Northern blot of reproductive tract tissues. The samples from individual surgical specimens are numbered. The location of molecular mass markers on the blot is indicated. The blot was hybridized with HBD-1 (*top*) and human actin (*middle*) cDNA as described in Materials and Methods. (*bottom*) The ratio of intensity of HBD-1 to actin mRNA bands in each lane is shown. Band intensities were determined by laser densitometer scan. Each grid line on the graph represents a 10-fold increase.

Characterization of recombinant HBD-1 peptides. Multiple forms of HBD-1 peptides were found that differed in NH_2-terminal truncation. The cDNA sequence indicates that HBD-1 is initially synthesized as a 68 amino acid peptide: MRTSYLLL FTLCLLLSEM ASGGNFLTGL GHRSDHYNCV SSGGQ-CLYSA CPIFTKIQGT CYRGKAKCCK. For convenience, the peptides will be referred to according to their number of amino acid residues (see Table I and Fig. 3). For the preparation of antibodies and initial studies of bioactivity, three recombinant forms of HBD-1 peptide were produced and purified to homogeneity, and characterized by NH_2-terminal sequencing and mass spectroscopy. The first HBD-1 (Fig. 3) contained 42 amino acid residues encoded at the COOH terminus of the HBD-1 cDNA and was the predominant secreted product of the insect cell line Sf21 infected by recombinant baculovirus containing HBD-1 cDNA. The second HBD-1 was three residues shorter at the NH_2 terminus (39 aa) and was the predominant secreted product when the High Five insect cell line (Invitrogen Corp., Carlsbad, CA) was infected with the same baculovirus construct. The third form of HBD-1 (36 aa) was produced by introducing a Ser-to-Met codon substitution into the HBD-1 cDNA to generate an artificial CNBr cleavage site, and the modified cDNA was expressed in baculovirus-infected insect cells. After treatment of the secreted product with CNBr, we obtained a peptide that matched the sequence and the expected mass of the 36 amino acid HBD-1 form isolated from human hemodialysate (16).

Detection of HBD-1 forms in urine, blood plasma, kidney, and vaginal lavage. Western blots of cationic proteins concentrated from urine specimens identified several immunoreactive peptides that migrated with similar electrophoretic mobilities as the rHBD-1 forms. The concentration of peptides in urine was estimated by Western blotting at 10–100 μg/liter ($n = 8$ donors) and the relative amounts of the various HBD-1 forms showed substantial interindividual variation (Fig. 4). Although there were multiple protein bands present, male urine contained only one predominant HBD-1 peptide as seen on Western blot, whereas urine from women generally contained two predominant bands. Pregnant women (G1 and G2) appeared to have higher levels of HBD-1 peptide than did the other subjects. Fig. 5 shows HPLC chromatograms of HBD-1 peptides from urine samples of two female donors. Four peaks were found to contain HBD-1 peptides (A–D) immunoreactive with anti–HBD-1 antibody. The fractions were characterized by mass spectrometry and NH_2-terminal sequencing (Table I), and found to contain HBD-1 forms with 40 aa (peak *A*), a mixture containing peptides with 36–43 (peak *B*), 44 (peak *C*), and 47 (peak *D*) aa. Although the relative amounts differed, peaks A and C were the predominant forms found in females and peak C was the predominant form found in males. The electrophoretic behavior of the purified forms is shown on Coomassie-stained gels and Western blots from acid-urea gels in Fig. 3. Although the 36 aa peptide is the smallest, it migrates slower than HBD-1 forms 39–44 aa in AU-PAGE since it has the fewest cationic residues. The remaining peptides migrate

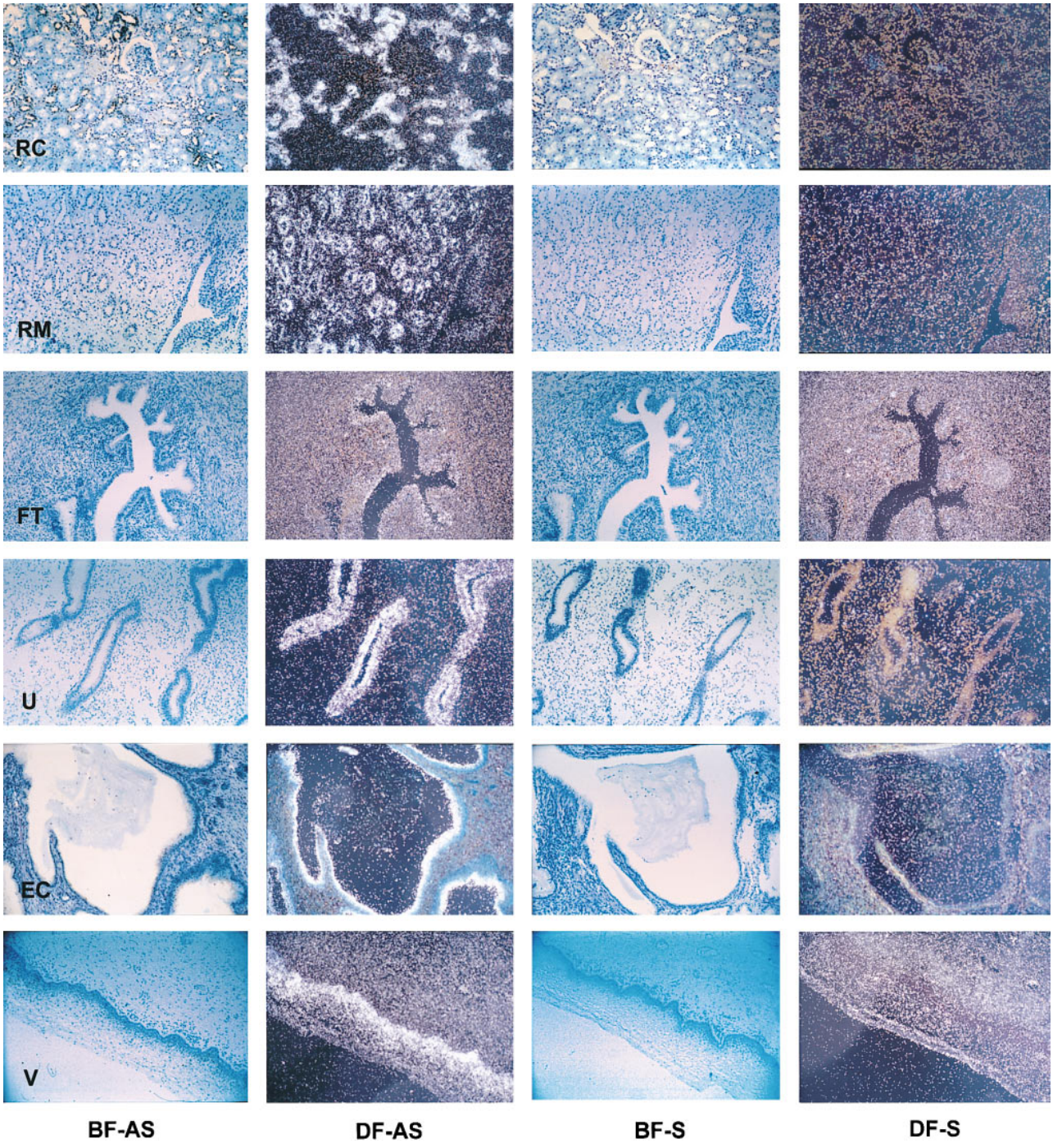

Figure 2. In situ hybridization of urogenital tissues. Tissue slides were hybridized with HBD-1 antisense (*AS*) and sense (*S*) 35S-labeled riboprobes and counterstained with toluidine blue. Bright field (*BF*) light microscopy shows tissue morphology, and dark field (*DF*) microscopy enhances the visualization of silver grains over areas of HBD-1 mRNA hybridization. Tissues shown are renal cortex (*RC*), renal medulla (*RM*), fallopian tube (*FT*), uterus (*U*), endocervix (*EC*), and vagina (*V*).

in the order expected from their size since they all have the same charge. To compare the conformation of natural and recombinant peptides, 33 amino acid fragments were generated by the cleavage of HBD-1 (40 aa) from urine and recombinant HBD-1 (39 aa) using AspN-endoproteinase. The resulting 33 aa peptides had identical conformations as judged by their HPLC retention time and electrophoretic mobility in AU-PAGE (data not shown). Furthermore, both were inactive in a CFU assay against *E. coli* ML-35p (data not shown).

Extracts of renal tissue (three kidneys, one of which was microdissected into cortex, superficial, and deep medulla) yielded HBD-1 concentrations similar to urine (i.e., ~50 ng/gram) with no significant differences between cortical and medullary samples. Analytical HPLC and Western blot (data

Table I. HBD-1 Peptides–Mass Spectroscopic Analysis

Deduced peptide	HPLC peak*	Mass (±0.1%) (MALDI-TOF)	Mass (mean±SD) (electrospray)	Mass (calculated)
$rHBD_{42}$-1			4535.3±1.6	4536.18
$rHBD_{39}$-1			4308.4±0.3	4308.92
$rHBD_{36}$-1		3929	3928.0±0.5	3928.52
HBD_{47}-1	D	5069	5068.7±0.7	5068.77
HBD_{44}-1‡	C	4750	4750.5±0.3	4750.45
HBD_{43}-1	B	4636	4637.1±1.3	4637.29
HBD_{42}-1	B	4536	4536.6±0.1	4536.18
HBD_{41}-1	B	4479	4479.1±0.4	4479.13
HBD_{40}-1	A	4366	4365.9±0.7	4365.97
HBD_{37}-1	B	4015	4015.3±0.9	4015.59
HBD_{36}-1	B	3929	3927.8±1.3	3928.52

*Peaks A, B, C, and D are the urinary HBD-1 peptides, see also Fig. 5.
‡NH_2-terminal sequence was determined as LTGL...

not shown) indicated that multiple forms of HBD-1 are present in the kidney tissue. Neutral pH blood plasma filtrate (10,000 mol wt cutoff) contained < 0.1 ng /ml of HBD-1 as assayed by dot blots, but an acidified plasma filtrate yielded ~ 0.2-1 ng HBD-1/ml plasma. The acid filtrate was analyzed by HPLC, AU-PAGE, and Western blot, and the predominant forms were identified as HBD-1 (44 aa) and HBD-1 (47 aa)

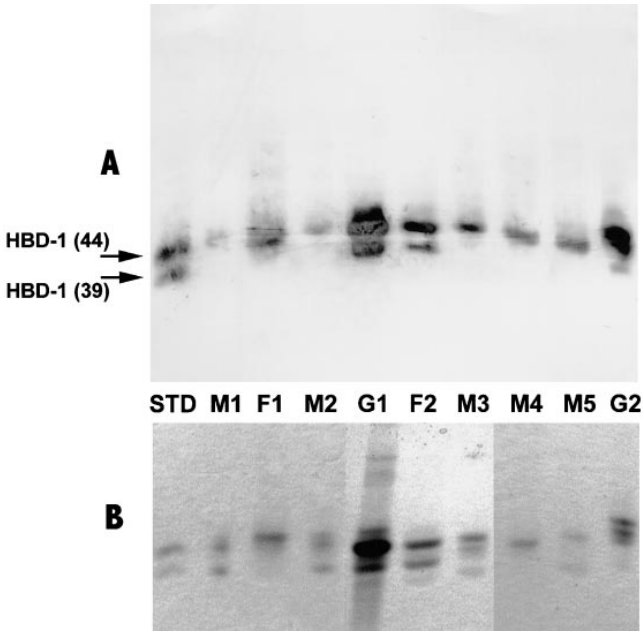

Figure 4. Western blot (*A*) and a duplicate AU-PAGE (*B*) of cationic protein fraction from urine specimens. The Western blot, on nitrocellulose, was probed with anti–HBD-1 serum. The lanes contain samples from individual male (*M*), nonpregnant female (*F*), or pregnant (gravid) donors (*G*). Each lane contains extract from 30 (Western) and 40 (AU-PAGE) ml urine. The standard lane contains 0.6 μg each of the 39 and 44 aa forms of HBD-1 (Western and AU-PAGE).

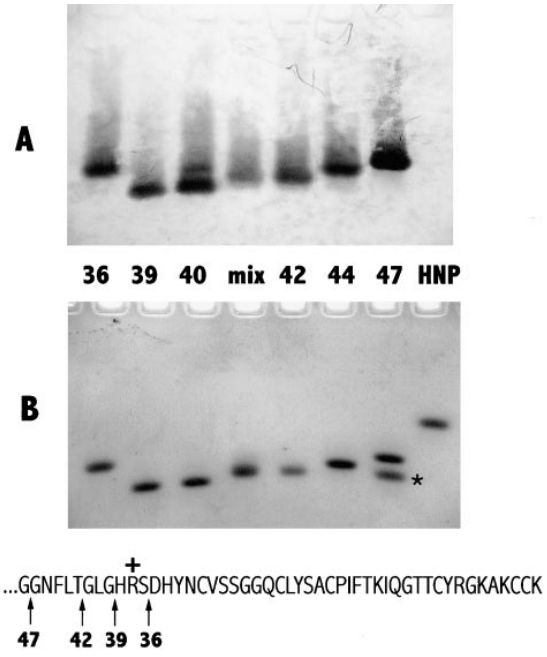

Figure 3. Western blot (*A*) and a duplicate AU-PAGE (*B*) of purified HBD-1 peptides (recombinant and native). The HBD-1 amino acid sequence is shown below with the various HBD-1 forms marked by arrows. A critical cationic residue removed from the 36 aa form is indicated (+); *an unrelated urinary peptide (present in this donor but not in others) that copurified with the 47 aa form of HBD-1 and was used as an internal negative control. Western blot lanes contain 60 ng peptide and are probed with anti–HBD-1 serum. For AU-PAGE, each lane contains 0.5 μg peptide.

(data not shown). Vaginal lavage fluid analyzed by HPLC and Western blot contained two predominant forms of HBD-1, sizes 39/40 and 44 aa at a total amount of ~ 150 ng/lavage (data not shown). The amount of peptide purified from the lavage was too small to size by mass spectrometry, and HBD-1 forms 39 and 40 aa are indistinguishable by HPLC and Western blot.

Immunolocalization of HBD-1 in tissue. Paraffin sections from the following urogenital tissues were stained with HBD-1

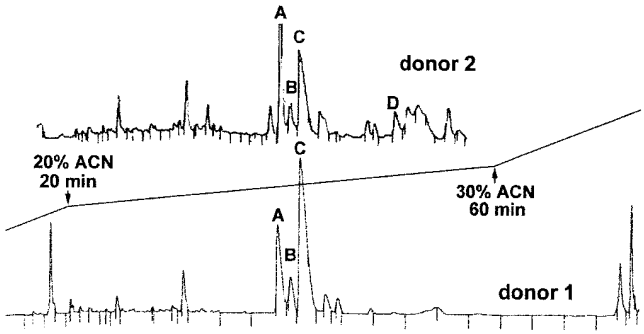

Figure 5. Reverse-phase HPLC fractionation of cationic peptides from human urine. The peptides were separated on a C18 Vydac 218TP54 column with a water/acetonitrile gradient (acetonitrile 0–20% in 20 min, 20–30% in 40 min, and then 40–60% in 20 min) with 0.1% trifluoroacetic acid as a pairing agent. Absorbance at 220 nm is shown. Downward event marks correspond to individual fractions. Peaks A–D contain HBD-1 forms (see Table I).

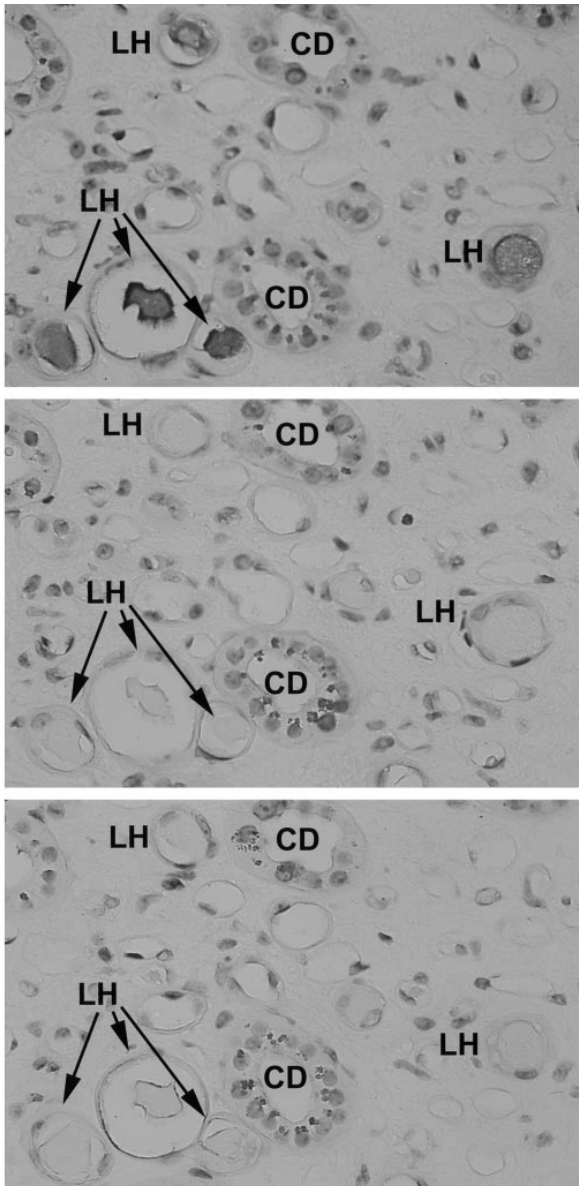

Figure 6. Immunostain of serial sections of the kidney. (*A*) Anti–HBD-1, (*B*) preimmune, (*C*) anti–HBD-1 absorbed with HBD-1. Antiserum was used at a dilution of 1:500. CD, collecting duct; LH, loop of Henle.

polyclonal antiserum—kidney, bladder, fallopian tube, endometrium, endocervix, ectocervix, and vagina. To accept tissue immunostain as evidence of HBD-1 accumulation, we required that staining be present with immune serum but absent with the same concentration of preimmune serum from the same rabbit, and absent with immune serum that had been immunosorbed with HBD-1. These criteria were fully satisfied only in the kidney, where there was specific staining of a hyaline substance in the lumen of the loops of Henle (Fig. 6). Similarly produced polyclonal antiserum against the neutrophil defensin HNP-1 intensely stained the cytoplasmic granules of neutrophils present in adjacent sections from the same tissues (not shown).

Antimicrobial Activity of HBD-1. The recombinant HBD-1 peptides were as active as the myeloid defensin HNP-2 against *E. coli* ML-35p in low ionic strength media but much less active against *Listeria monocytogenes* (data not shown). The activity of rHBD-1 (39 aa) in 10 mM $NaPO_4$ buffer was not affected by lowering the pH from 7.4 to 5.5, but it was inhibited by raising the NaCl concentration to 100 mM (data not shown). To mimic the milieu of the renal tubules, we tested the antimicrobial activity of HBD-1 against *E. coli* (laboratory strain ML-35p, and a fresh clinical isolate from a urinary tract infection) in urine collected after high volume water intake (low salt urine). This procedure was designed to inhibit the concentrating mechanisms in the collecting duct that modify the urine after its transit through the distal convoluted tubules. For comparison, antimicrobial activity was also tested in 10 mM sodium phosphate, pH 7.5, and higher salt urine. As seen in Fig. 7, of the recombinant peptides, the 36 aa form of HBD-1 was the most potent in all of the conditions tested and remained active even under high salt conditions. The 42 aa form was potentiated in low salt urine but was inhibited by high salt urine. Activity in urine was not due to the recombinant mode of production since 42 aa HBD-1 that could be isolated in small amounts from the urine of one donor was as active by radial diffusion assay (32) as the recombinant 42 aa HBD-1 (data not shown). The 39 aa form retained its activity against *E. coli* ML-35p in low salt urine but was inhibited by high salt urine. Peptides 40 and 44 aa HBD-1 isolated from urine were inhibited when tested in urine. In general, the HBD-1 peptides were as potent against both types of *E. coli,* except that forms 39 and 40 aa were virtually inactive against the clinical *E. coli* isolate. At 30 μM, the highest concentration tested, 47 aa HBD-1 was active in both high and low salt urine. Thus, all forms of HBD-1 exhibit antimicrobial activity but have different profiles of activity under the various conditions tested.

Discussion

The mechanisms that protect genitourinary organs from ascending infection by microbes present in the perineum and the vagina are only partially understood. Since most mucosal surfaces do not normally contain abundant phagocytic cells, one would expect that epithelial cells and their secretions directly interfere with microbial colonization and invasion. Indeed, human mucosal epithelia secrete antimicrobial proteins and peptides, which include lysozyme, lactoferrin, and secretory leukoprotease inhibitor, in addition to defensins (5–8, 33–40).

In this study, we detected abundant mRNA for HBD-1 in the kidney and in the epithelium-enriched tissue fragments from the endocervix of the female genital tract. By in situ hybridization, HBD-1 mRNA was localized to the epithelial layers of the loops of Henle, the distal tubules, and the collecting ducts in the kidney and throughout the female genital tract. Although HBD-1 mRNA can be detected in many other epithelial tissues by reverse transcriptase–PCR (18), the concentrations of HBD-1 mRNA in the urogenital tissues greatly exceeded those in other organs and tissues. The corresponding HBD-1 peptide forms were identified in urine, kidney, blood plasma, and vaginal lavage.

Multiple forms of the HBD-1 peptide were found, reflecting variable NH_2-terminal proteolytic processing. The two insect cell lines differed in the processing of the secreted forms, indicating that these cell types recognize distinct HBD-1 pro-

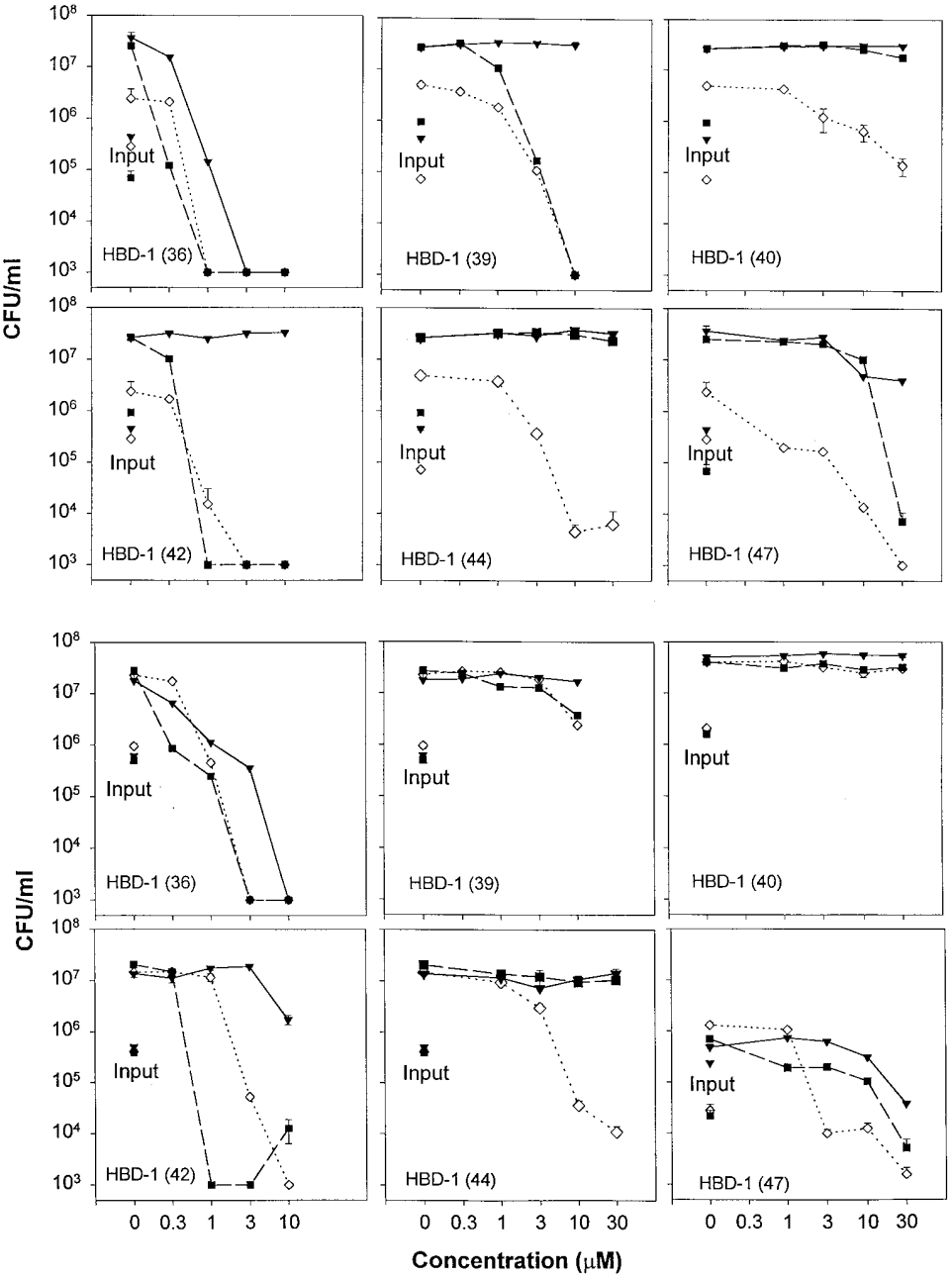

Figure 7. CFU antimicrobial peptide assays of HBD-1 activity against *E. coli* ML-35p (*top*) and a clinical isolate of *E. coli* (*bottom*). Each square contains plots of the mean±standard error of triplicate CFU determinations on the vertical axis, at various concentrations of peptide shown on the horizontal axis. Assay conditions: G, 10 mM sodium phosphate, pH 7.4, and conductance 1.58 mS; ■, low salt, unconcentrated urine, pH 7.1, and 1.09 mS; ▼, higher salt, normal urine, pH 6.5, and 9.2 mS. For reference, input CFU counts are also shown (at the start of 3 h incubation at 37°C).

cessing sites. The form of HBD-1 peptide originally isolated from human hemodialysate from patients with chronic renal failure (16) corresponds to the most extensively processed (36 amino acid) HBD-1 peptide in urine. Although other previously characterized prodefensins (α-defensins) were inactive until processed to mature forms (27), all the HBD-1 peptides were antimicrobial. This suggests that, unlike the α-defensins stored in granules, the β-defensin peptides do not contain an inhibitory proregion. Nevertheless, relatively minor NH_2-terminal changes in HBD-1 peptides (e.g., the presence of glycine on the 40 aa form as compared with the 39 aa form) had a marked effect on HBD-1 microbicidal activity, indicating that the amino terminus is functionally important. This conclusion is further supported by the inactivity of the 33 aa HBD-1 generated by AspN-endoproteinase cleavage from both 39 aa recombinant and 40 aa native forms of HBD-1. The NH_2-terminal processing of HBD-1 and other defensins may be a mechanism for generating functional diversity.

Indirect evidence for the microbicidal function of HBD-1 was provided by recent studies of human epithelial cells maintained in nude mice, wherein the antimicrobial activity of epithelial secretions was ablated by antisense oligonucleotides directed against HBD-1 mRNA, but not by control oligonucleotides (19). In our experiments, the various forms of HBD-1 were microbicidal against *E. coli,* suggesting that they could function as antimicrobial agents in urogenital epithelial host defense. Although in healthy volunteers, the aggregate concentrations of HBD-1 detected in urine and vaginal fluid were probably too low to be directly antimicrobial, peptide production may be inducible by appropriate stimuli, higher peptide

concentrations of HBD-1 are likely near the sites of peptide secretion and, in addition, the peptides may be further concentrated on the negatively charged epithelial surfaces. The low salt conditions that prevail in distal convoluted tubules are highly favorable to defensin activity. Moreover, even compared with low salt buffer, the microbicidal activity of the 42 aa form of HBD-1 was further potentiated by urine, pointing to the existence of other factors that may act synergistically with HBD-1. Further studies will be necessary to determine whether HBD-1 exhibits such additional defensin activities as chemoattraction for mononuclear cells, stimulation of epithelial secretion, inhibition of fibrinolysis, and inhibition of cortisol production in the adrenal gland (1).

In the urogenital tissues examined, the luminal contents of loops of Henle were the only structures whose staining with anti–HBD-1 antibody exceeded the nonspecific background (Fig. 6). Since the anti–HBD-1 antibody stained nanogram amounts of all natural and recombinant HBD-1 forms in formalin-fixed Western blots, and since similarly produced antibodies against human defensins HNP1–3 and HD-5 specifically stain the cytoplasmic granules of neutrophils and Paneth cells, respectively (7, 41), we suspect that the HBD-1 peptide is secreted without intracellular storage. An analogous situation exists in the bovine tracheal epithelium, where the tracheal antimicrobial peptide (a β-defensin) is secreted by epithelia that lack visible cytoplasmic granules. We considered and could not exclude the possibility that some HBD-1 peptides in urine originate, at least in part, from plasma filtrate. However, to appear in urine, HBD-1 would have to escape the efficient peptide absorption mechanism in the proximal tubule (42, 43). Women have two predominant forms of HBD-1 (40 and 44 aa) in the urine, and the same forms were found in the vaginal secretions. There was $\sim$ 150 ng HBD-1 present in the vagina at the time of the lavage. Since only a small portion of the vaginal secretions could be transferred into the urine during micturition, it is unlikely that this source of peptide contributes substantially to urinary HBD-1 levels. These considerations favor the conclusion that local production of HBD-1 by the epithelial lining cells of the distal nephron is the predominant source of HBD-1 in urine.

The observed interindividual variability of HBD-1 mRNA levels in tissue and peptide levels in urine may reflect the physiologic regulation of HBD-1 synthesis and release. The mRNAs for the two known mucosal epithelial β-defensins, bovine TAP and LAP, accumulate in epithelia after local trauma or exposure to microbial products, due to direct or cytokine-mediated transcriptional induction (10, 12). Similar stimuli may also induce HBD-1 synthesis. The vagina and cervix are subject to infections with sexually transmitted pathogens as well as microtrauma from sexual intercourse and tampon use, and these may influence the local expression of HBD-1. Other influences that may regulate HBD-1 production include estrogen and progesterone, previously reported to modulate the concentration of the antimicrobial proteins lactoferrin and lysozyme (38, 44–46) in the cervix and vagina. Observations in our small sample were consistent with such an effect (Fig. 4), where we noted the highest concentrations of HBD-1 in the urine of pregnant women, intermediate concentrations in nonpregnant women, and the lowest concentrations in men. The effects of temporal or individual variations in mucosal defensin levels on susceptibility to ascending infections of the urogenital tract remain to be determined.

Acknowledgments

We dedicate this manuscript to the memory of Sylvia Harwig, who helped develop much of the technical foundation for this work and whose premature death is a loss that continues to be felt by all who worked with her. We gratefully acknowledge the many helpful discussions and unpublished data shared with us by Drs. Robert Lehrer, Rose Linzmeier, and Lide Liu. We thank Dr. Clark Robbins for providing us with essential tissue samples, Sylvia Harwig, Daniel Martin, and Nhu Dinh for their excellent assistance with HPLC purifications, and Dr. Kym Faull (UCLA Mass Spectrometry Facility) and Dr. Audrey Fowler (UCLA Protein Sequencing Facility) for their help with the characterization of the urinary peptides.

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