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Broad-Spectrum Antimicrobial Activity of Human Intestinal Defensin 5

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Defensins are antibiotic peptides expressed in human and animal myeloid and epithelial cells. Due to the limited availability of natural peptides, the properties of human epithelial defensins have not been studied. We assayed the microbicidal activity of recombinant human intestinal defensin 5 (rHD-5) in the presence of salt (0 to 150 mM NaCl) with varied pH (pH 5.5 to pH 8.5) and trypsin (25 and 250 μg/ml). rHD-5 exhibits microbicidal activity against *Listeria monocytogenes*, *Escherichia coli*, and *Candida albicans*. In contrast to cryptdins, the mouse intestinal defensins, rHD-5 is active against both mouse-virulent wild-type *Salmonella typhimurium* and its isogenic, mouse-avirulent *phoP* mutant. In the presence of salt, rHD-5 activity was reduced, and at 100 mM NaCl, activity against *S. typhimurium* was abolished. However, at all salt concentrations tested, rHD-5 remained bactericidal to *L. monocytogenes*. Activity against *L. monocytogenes* was not pH dependent but was diminished at pH 5.5 against wild-type *S. typhimurium*. This acid-induced resistance may have been mediated by the virulence gene regulator *phoP*, since the *phoP* mutant was equally sensitive at pH 5.5 and pH 7.4. In the presence of trypsin, rHD-5 was partially cleaved, but even then, rHD-5 at 100 μg/ml decreased the number of CFU of wild-type *S. typhimurium* by more than 99%. The persistence of microbicidal activity of rHD-5 under these conditions supports the notion that naturally occurring human intestinal defensin is an effective arm of mucosal host defense.

Mucosal host defenses include a number of antimicrobial proteins and peptides that are either continuously present or produced and secreted upon microbial challenge. Antimicrobial proteins of mucosal secretions (defined as polypeptides with sizes of >10 kDa) include lactoferrin, lysozyme, and phospholipase A_2 (11, 25, 34, 39, 43). Antimicrobial peptides (defined as those with sizes of <10 kDa) include the magainins of amphibian skin (6, 8) as well as the defensins in the mucosal secretions of mammals (13, 14, 16, 44, 45, 47, 48, 51). In human mucosae, mRNA of intestinal defensins 5 and 6 (HD-5 and HD-6) were localized in Paneth cells (26, 27), and the human β-defensin 1 (HBD-1) purified from human hemodialysate (5) was found to be expressed in urogenital tissue (55). Defensins (for reviews, see references 20, 32, and 35) are cationic, arginine-rich, small peptides between 3.5 and 4 kDa in size with six cysteines that form three disulfide bridges. They bind electrostatically to negatively charged membranes, multimerize, and form pores. By these and subsequent as-yet-uncharacterized mechanisms, defensins exert antimicrobial activity against gram-positive and gram-negative bacteria, *Mycobacterium tuberculosis* (40), *Treponema pallidum*, *Chlamydia trachomatis* (58), *Candida* spp. and other fungi, and enveloped viruses. Cryptdins, the mouse intestinal defensins, have been isolated and found to be active against *Listeria monocytogenes*, *Escherichia coli*, and *Giardia intestinalis* (1, 16, 44, 45, 51). Cryptdins are substantially less active against mouse-virulent wild-type *Salmonella typhimurium* than against the mouse-avirulent isogenic *phoP* mutant (16, 45, 51).

Human intestinal defensins have not been isolated and characterized yet. Because of the limited availability of fresh human tissue, we biosynthesized HD-5 in the baculovirus-insect cell system. We then used the recombinant HD-5 (rHD-5) to produce polyclonal antibodies and immunolocalized HD-5 in the granules of Paneth cells. In this study we characterized the antimicrobial spectrum of rHD-5 and explored the effects of salt concentration, pH, and trypsin on its antibacterial activity.

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MATERIALS AND METHODS

Biosynthesis and purification of rHD-5. rHD-5 was prepared as described in an accompanying publication (45a).

Antimicrobial assays. *L. monocytogenes* EGD, *E. coli* ML35p (ampicillin-resistant strain) (31), *S. typhimurium* 14028S (mouse-virulent wild type), *S. typhimurium* 7953S (isogenic mouse-avirulent *phoP* mutant) (for reviews, see references 22 and 37) and *C. albicans* 820 were kindly provided by Robert I. Lehrer, (UCLA School of Medicine, Los Angeles, Calif.). *L. monocytogenes* and *C. albicans* were maintained on Columbia-colistin nalidixin agar (CNA) plates with 5% sheep erythrocytes (Becton Dickinson, San Jose, Calif.), and the gram-negative bacteria were maintained on Trypticase soy agar (TSA) plates (Clinical Standards Laboratories, Rancho Dominguez, Calif.) without supplement (*S. typhimurium* 14028S) or supplemented with 100 μg of ampicillin per ml (*E. coli* ML35p) or 50 μg of tetracycline per ml (*S. typhimurium* 7953S). Single cell colonies were cultured overnight in 3% Trypticase soy broth (TSB), subcultured at 1:1,000 (gram-negative bacteria) or 1:100 (*L. monocytogenes*) in prewarmed TSB, and grown for 2.5 h to mid-exponential growth phase in a 37°C shaking incubator. The subcultures were centrifuged for 10 min at 4°C at 900 × *g*, and the bacterial pellets were washed once with 10 mM sodium phosphate (pH 7.4) (Na_2HPO_4/NaH_2PO_4)–1% TSB. The bacterial concentration was estimated photometrically (an optical density at 620 nm of 0.2 is ~5×10^7 bacteria/ml), and working dilutions of 10^6 bacteria/ml in 10 mM sodium phosphate (pH 7.4)–1% TSB were prepared. *C. albicans* was used in stationary growth phase, and overnight cultures were processed as described for the bacterial subcultures (the optical density at 450 nm of 1 is ~2.87×10^7 yeast cells/ml).

For the CFU assay, 10 ml of 10× stock concentrations of rHD-5 or human neutrophil defensin 2 (HNP-2) (21) in 0.01% acetic acid, or 10 μl of the solvent only, were mixed with 90 μl of the bacterial suspension. To determine the initial number of CFU, the incubation mix was immediately diluted 100-fold with 10 mM sodium phosphate (pH 7.4) and kept on ice (t_0 value) until plating. The remaining samples were incubated at 37°C in a shaking water bath for 3 h and then diluted 100-fold in 10 mM sodium phosphate (pH 7.4). All dilutions were plated in triplicate on TSA plates (Clinical Standards Laboratories) by using a spiral plater (Spiral Systems, Inc., Cincinnati, Ohio), which deposits a defined volume per area. In addition, 15 μl of undiluted material from each assay tube was plated manually in duplicate on TSA plates to detect very low numbers of surviving bacteria. The plates were incubated 12 to 16 h at 37°C, the colonies

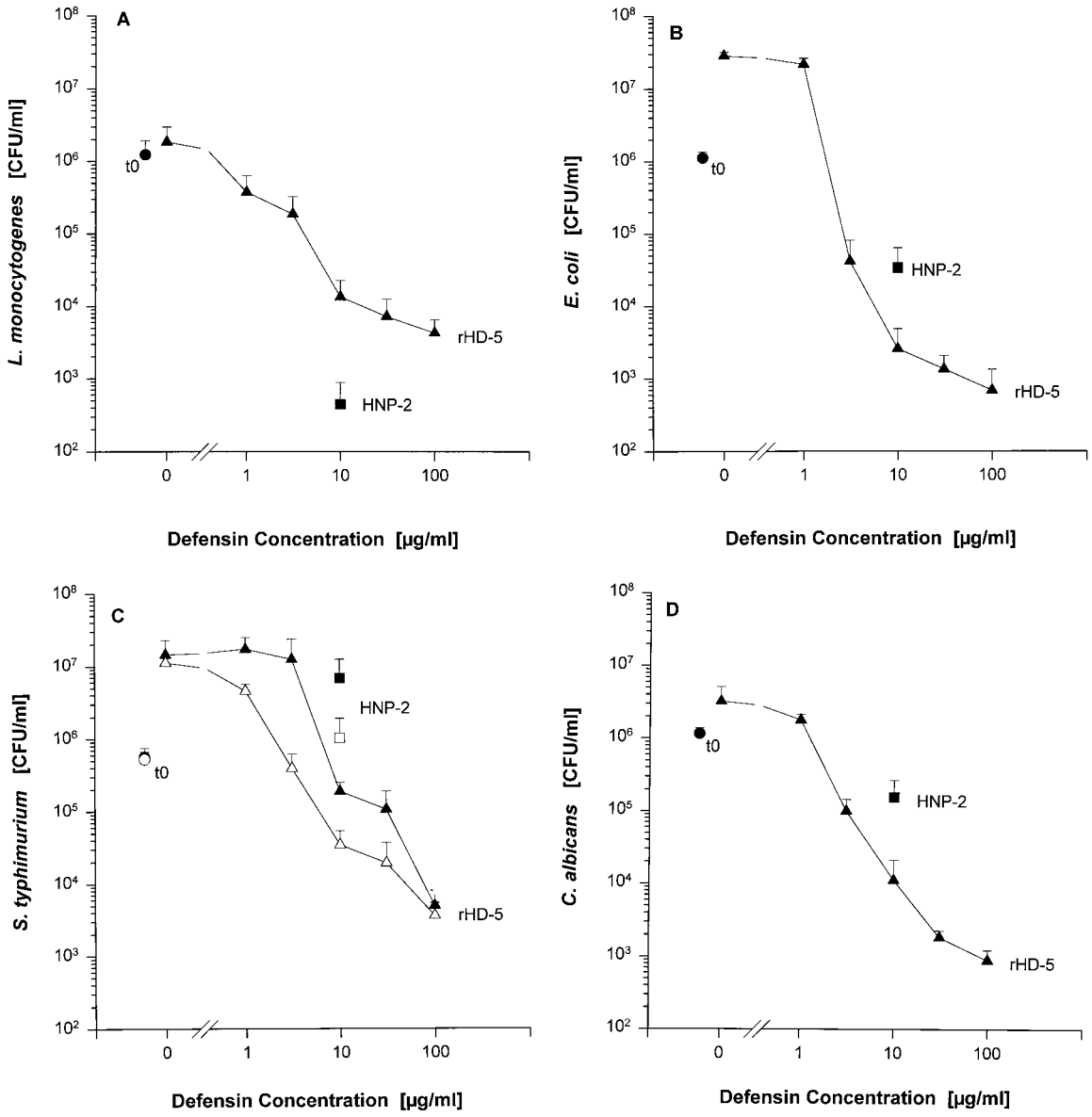

FIG. 1. Activity of rHD-5 against *L. monocytogenes* (A), *E. coli* ML35p (B), *S. typhimurium* (wild type, closed symbols; *phoP* mutant, open symbols) (C), and *C. albicans* (D) by CFU assay. The number of CFU was determined in triplicate after 3 h of incubation with rHD-5 or HNP-2. For the time zero controls (t0), aliquots of microorganisms mixed with peptide solvent were diluted and immediately incubated for 3 h on ice. The means ± SEMs (error bars) for three experiments are shown.

were counted, and the number of CFU per ml was calculated. In order to assess the effect of salt or pH on rHD-5 activity, the sodium phosphate buffer was either supplemented with 25 to 150 mM NaCl or prepared at pH 5.5 or pH 8.5. For analysis of the effect of trypsin on the bactericidal activity of rHD-5, bacteria were diluted to 1.25×10^6 CFU/ml, and 80 μl of the bacterial suspension was mixed with 10 μl of 10× rHD-5 solution or solvent and 10 μl of a 10× trypsin stock solution in 10 mM sodium phosphate (pH 7.4)–1% TSB or solvent.

The radial diffusion agar assay was performed as described by Lehrer et al. (33).

Trypsinization of rHD-5. To analyze the cleavage of rHD-5 by trypsin, defensin was incubated with the enzyme as described for the antimicrobial assay but without bacteria. Replicate samples were kept at −20°C. To inactivate trypsin, 1 volume of 2% acidic acid was added to the samples followed by dialysis against 2% acidic acid by using a 1,000-molecular-weight-cutoff membrane (SpectraPor; Spectrum, Houston, Tex.). Samples were lyophilized, subjected to acid-urea–polyacrylamide gel electrophoresis (AU-PAGE), and stained with Coomassie blue (without formalin). The bands corresponding to rHD-5 and its cleavage product were cut out, transferred to Eppendorf tubes, pulverized, lyophilized, subjected to reducing sodium dodecyl sulfate (SDS)-Tricine PAGE, and silver stained for detection of cleavage products without interference from disulfide bonding.

Data analysis. The Sigmaplot (Jandel Scientific, San Rafael, Calif.) program was used to calculate the mean and the standard error of mean (SEM) for each datum point and to prepare the corresponding graphs. PC-Gene (Intelligenetics, Inc., Palo Alto, Calif.) software was used to calculate the charge of rHD-5 at various pH values.

RESULTS

HD-5 is a broad-spectrum antibiotic. In a concentration-dependent manner, rHD-5 was microbicidal to all bacterial strains and *C. albicans* (Fig. 1). In contrast to HNP-2, rHD-5 was also active against the mouse-virulent *S. typhimurium* wild-type strain (Fig. 1C). At the lowest concentration of 1 μg/ml,

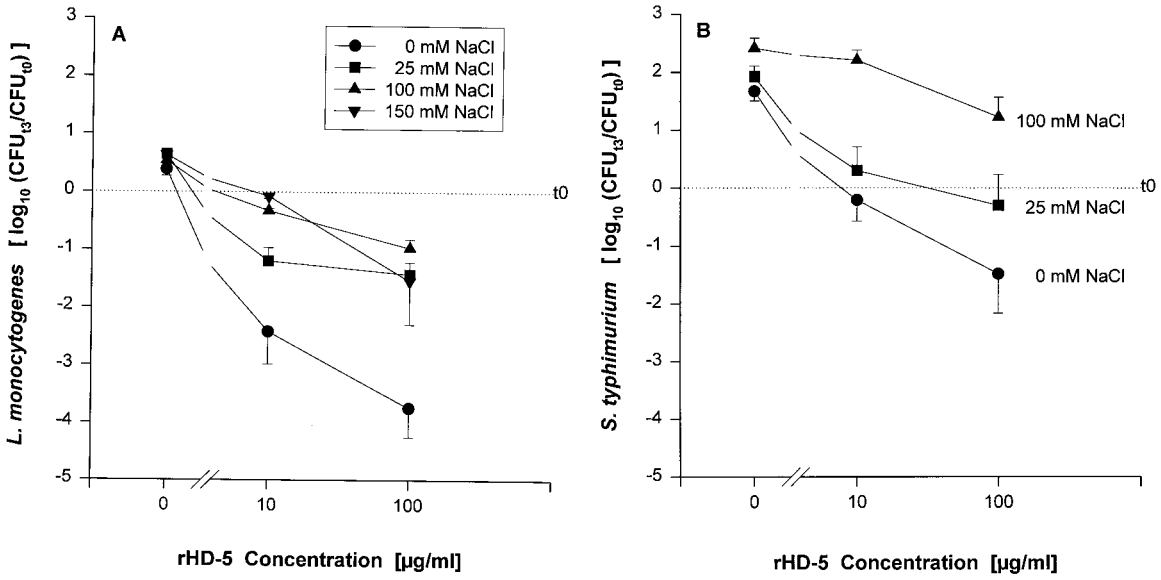

FIG. 2. Effect of salt on antibacterial activity of rHD-5. The number of CFU was determined in triplicate with *L. monocytogenes* (A) and wild-type *S. typhimurium* (B) after 3 h of incubation on ice ($t0$) or at 37°C ($t3$) and expressed as ($\log_{10}$ CFU_{t3}/CFU_{t0}). The means ± SEMs (error bars) for three experiments are shown.

rHD-5 was bactericidal to only *L. monocytogenes* (Fig. 1A). At 10 μg/ml, rHD-5 was more active than HNP-2 against all targets except *L. monocytogenes.*

rHD-5 activity against wild-type *S. typhimurium* is confirmed by radial diffusion assay. The radial diffusion assay was employed to verify human intestinal defensin activity against the mouse-virulent *S. typhimurium* strain and to be able to compare the data for rHD-5 activity with the published data on murine intestinal defensins (cryptdins) (16, 51). Because rabbit neutrophil defensins are active against wild-type *S. typhimurium* (16, 51), we used NP-2 as a positive control. Results from the CFU assay were confirmed, showing that rHD-5 is active against both the defensin-sensitive *phoP* mutant and the wild-type strain, with higher activity against the *phoP* mutant strain (data not shown). NP-2 was more active than rHD-5 against both the wild type and the *phoP* mutant, but the *phoP* strain was again more sensitive than wild-type *S. typhimurium.* Eisenhauer et al. (16) and Selsted et al. (51) both documented complete resistance of the wild-type strain to cryptdin as opposed to the cryptdin sensitivity of the *phoP* mutant strain.

Antibacterial activity of rHD-5 is inhibited by salt. To evaluate the salt dependency of intestinal defensin the CFU antimicrobial assay was performed at concentrations of 25, 100, and 150 mM sodium chloride. Activity of rHD-5 was reduced against both strains in the presence of salt, but there was less inhibition of activity against *L. monocytogenes* (Fig. 2A) than against wild-type *S. typhimurium* (Fig. 2B). At 100 mM sodium chloride and a concentration of 100 μg/ml, rHD-5 was still bactericidal to *L. monocytogenes*, whereas it was only growth inhibitory to *S. typhimurium*.

rHD-5 activity is maintained throughout a broad pH range. Activity of rHD-5 was assessed at pH 5.5, pH 7.4, and pH 8.5. Since the growth kinetics of bacteria during the 3-h test incubation time were affected by pH (data not shown), the test incubation time was reduced to 1 h for this set of experiments. For *L. monocytogenes* rHD-5 activity was maintained, effecting at least a 99% decrease in CFU at each pH tested (Fig. 3A). For wild-type *S. typhimurium* (Fig. 3B), the activity of rHD-5 at pH 8.5 did not differ from the activity observed at pH 7.4. In contrast, at pH 5.5 defensin acivity was reduced, even though bactericidal action was still detectable.

Increased resistance towards rHD-5 at acidic pH is observed in wild-type *S. typhimurium* but not in the *phoP* mutant. To determine whether increased resistance towards rHD-5 at acidic pH might be linked to acid-mediated induction of virulence characteristics of wild-type *S. typhimurium*, rHD-5 (100 μg/ml) was tested at pH 7.4 and pH 5.5 against the wild type and the isogenic nonvirulent *phoP* mutant. In contrast to the wild type, the sensitivity of the *phoP* mutant to rHD-5 was not reduced at pH 5.5; the $\log_{10}$ decreases in CFU for the wild type and *phoP* mutant were (means ± SEMs) 2.8 ± 0.12 and 3.2 ± 0.45 at pH 7.4 and 0.96 ± 0.36 and 3.2 ± 0.15 at pH 5.5 (n = 2), respectively.

rHD-5 is relatively stable in the presence of trypsin. In the small intestine, polypeptides are exposed to the action of proteolytic enzymes. We analyzed the effect of trypsin, one of the major proteases which cleaves at basic amino acid residues, on the arginine-rich rHD-5. However, after a 3-h incubation at 37°C in the presence of 25 or 250 μg of trypsin per ml, rHD-5 was only partially cleaved at the high concentration of the enzyme. AU-PAGE revealed an additional, minor band, which migrated faster than rHD-5 (Fig. 4A). To exclude the possibility that stabilizing disulfide bridges mask trypsin cleavage, all rHD-5 bands were cut out of the AU gel and subjected to reducing SDS-Tricine-PAGE (Fig. 4B). However, even under reducing conditions, all rHD-5 bands still showed identical sizes. The band corresponding to the faster-migrating fragment had an estimated molecular mass of 2,000 Da. In agreement with the observed resistance of rHD-5 to trypsin cleavage, the bactericidal activity of rHD-5 against virulent wild-type *S. typhimurium* was unchanged at the low trypsin concentration and somewhat decreased at the high concentration of the enzyme (Fig. 4C). The activity of rHD-5 against *L. monocytogenes* in the presence of trypsin could not be assessed because trypsin itself was bactericidal to *L. monocytogenes.*

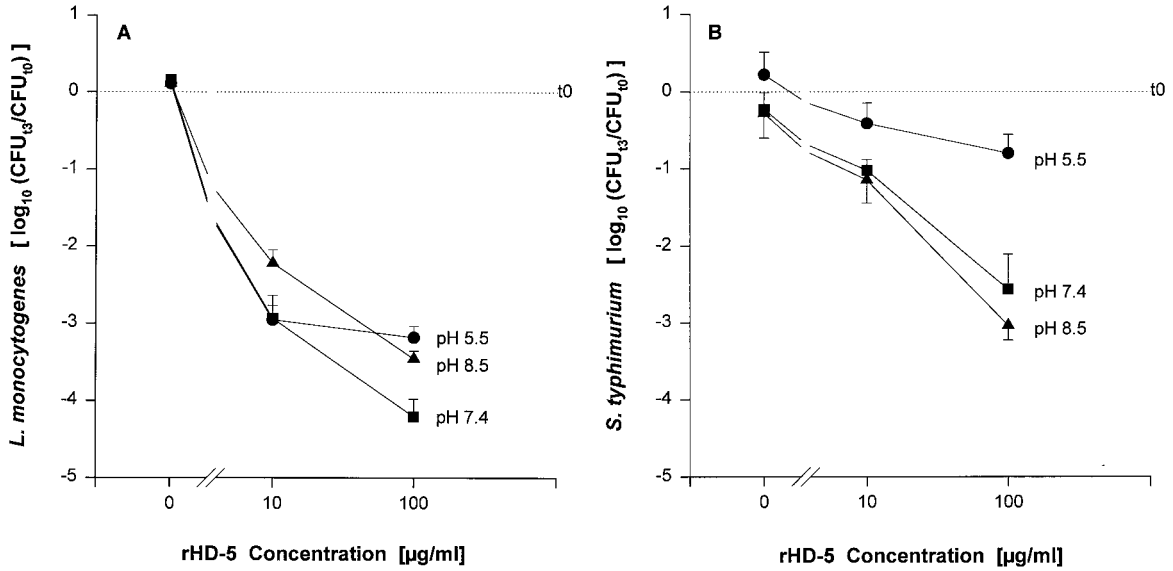

FIG. 3. Effect of pH on antibacterial activity of rHD-5. The number of CFU was determined after incubation with rHD-5 or peptide solvent for 1 h on ice ($t0$) or at 37°C ($t1$) and expressed as ($\log_{10}$ CFU_{t1}/CFU_{t0}). The means ± SEMs (error bars) for three experiments with *L. monocytogenes* (A) and wild-type *S. typhimurium* (B) are shown.

DISCUSSION

This study explored the antimicrobial activity of human intestinal defensin under various conditions that could be encountered in the small intestinal milieu. rHD-5 was active at low concentrations against gram-positive and gram-negative bacteria and the fungus *C. albicans* in a manner similar to that of other mammalian defensins (16, 17, 33, 41, 46, 49, 51, 52).

The antibacterial activity of rHD-5 was found to be inhibited in the presence of salt, an effect which has also been reported for HNP-1 to HNP-3 and NP-1 (41, 52). The initial binding of cationic defensins to their anionic target membranes is thought to depend on an electrostatic interaction (57) that is vulnerable to increased salt concentrations and is followed by the formation of voltage-dependent ion channels (28). The effects of salt on the interaction of bacterial surfaces with defensins were confirmed by Shimoda et al. (53), who reported that ultrastructural changes on the *S. aureus* surface caused by defensin were prevented at high-salt concentrations.

However, the binding and activity of defensins must involve additional specific interactions with the surface of the target organism that are less affected by salt. Salt-resistant activity of HNP-1 was seen with *M. tuberculosis* (40), a bacterium with a unique cell wall of remarkably high lipid content, and various enveloped viruses (12) whose viral envelopes are derived from the host's cell membrane. In this study, rHD-5 was bactericidal against *L. monocytogenes* at physiological salt concentrations but could not reduce the number of *S. typhimurium* CFU below that of the inoculum. However, the comparison of rHD-5 activities against *L. monocytogenes* versus *S. typhimurium* was complicated by the higher growth rate of *S. typhimurium*, which was even further enhanced in the presence of salt.

Paneth cells, which produce HD-5, and the sites of initial invasion by *S. typhimurium* and *L. monocytogenes* are located in the small intestine, where the pH changes from pH 5 to pH 8 from the proximal to the distal end (18, 19, 36, 56). These changes in pH could affect the activity of rHD-5 by modifying either the peptide or its target. The net charge of antimicrobial peptides parallels their activity (7, 52) and could be influenced by the pH depending on the amino acid composition of the peptide. For rHD-5 the charge is not altered from pH 5.5 to pH 8.5 (Chargepro; PC Gene) (data not shown), and we did not expected rHD-5 activity to be influenced by the pH unless the properties of the bacterial targets were modified by pH changes. We found that the activity of rHD-5 against *S. typhimurium* was reduced at pH 5.5 in contrast to the activity against *L. monocytogenes*, which was maintained at each pH tested. The ambient pH has been shown to influence the DNA topology of the *S. typhimurium* genome (29) and the expression of mouse-virulence genes (3). In particular, a low pH induces *phoP*-activated virulence genes (2, 4), some of which mediate defensin resistance (24, 38). Consequently, we tested the rHD-5 sensitivity of the *phoP*-defective mutant at pH 5.5 and observed that the mutant's defensin sensitivity was unaffected, supporting the hypothesis that resistance of wild-type *S. typhimurium* to rHD-5 at pH 5.5 is increased due to induction of *phoP*.

To our knowledge, the effects of physiologic proteases on defensin structure and activity have not been reported. Exhaustive protease treatment was successfully employed for peptide fragmentation to assist in the sequence and structure analysis of HNP-2 (50) and the bovine neutrophil β-defensin BNBD-12 (54). As an intestinal peptide, HD-5 potentially encounters many proteases in luminal fluids, and Paneth cells have also been reported to contain trypsin (9). We tested rHD-5 stability in the presence of trypsin, which cleaves at basic amino acid residues and thus would be expected to be particularly effective against the arginine-rich rHD-5. Incubation with rHD-5 was conducted at physiological concentrations of trypsin (compare with measured levels [15, 30]) but without the inhibitors normally found in the intestine. Under these conditions rHD-5 was relatively stable, as shown by PAGE under nonreducing and reducing conditions. Although partial cleavage of rHD-5 diminished the activity against *S. typhimurium*, greater than 99% killing was still seen at higher rHD-5 concentrations. The rigid cysteine-bridging motif of classical defensins (50) possibly blocks the access of trypsin to susceptible sites. In vivo, intracellular and secreted rHD-5 may

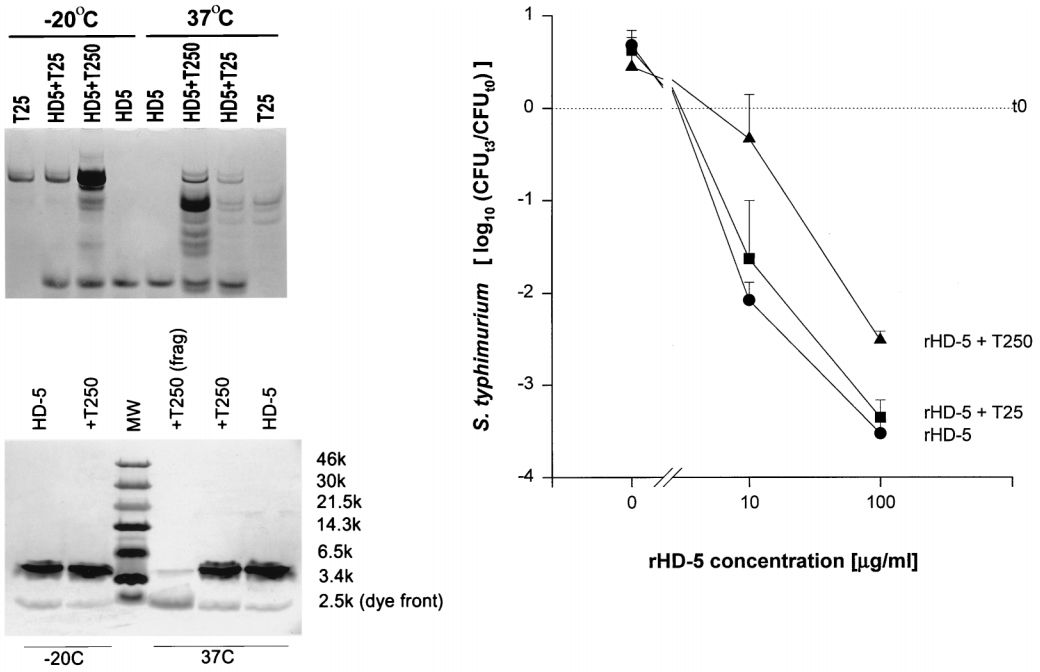

FIG. 4. Effect of trypsin on rHD-5. (A) AU-PAGE of trypsin-treated rHD-5. rHD-5 (10 μg/ml) was incubated in 10 mM sodium phosphate buffer (pH 7.4) containing 1% TSB with 25 (T25) or 250 (T250) μg of trypsin/ml or without trypsin for 3 h at 37 or −20°C. The reaction was stopped with 2% acidic acid, and samples were dialyzed against 2% acidic acid, lyophilized, and subjected to AU-PAGE. (B) Bands corresponding to rHD-5 and its cleavage product were cut out of the AU gel, subjected to SDS-Tricine PAGE, and silver stained. The lane corresponding to the rHD-5 fragment shows a minor contamination with uncleaved rHD-5 and the appearance of a protein band of approximately 2 kDa near the dye front. (Molecular sizes in kilodaltons are indicated on the right.) Digital images were obtained with the Speed Light Gel Documentation system (manual version 1.00; Lightools Research, Encinitas, Calif.) and PhotoStyler 2.0 software (Aldus Corporation, Seattle, Wash.). (C) Activity of rHD-5 against wild-type *S. typhimurium* in the presence of trypsin. Bacteria were suspended in 10 mM sodium phosphate buffer (pH 7.4) containing 1% TSB unsupplemented (●) or supplemented with trypsin (25 [■] or 250 [▲] μg/ml), mixed with 10-fold stock solutions of rHD-5 or peptide solvent, and incubated for 3 h at 37°C (t3). For the controls at time zero (t0), aliquots of bacteria without trypsin mixed with peptide solvent were diluted and immediately incubated for 3 h on ice. Thereafter, the number of CFU was determined in triplicate, and the results, expressed as ($\log_{10}$ CFU_{t3}/CFU_{t0}), are means for three independent experiments; error bars indicate SEMs.

be further protected by trypsin inhibitors, which have been also detected in Paneth cells (10, 23, 42).

The surprising persistence of rHD-5 microbicidal activity under various conditions that may occur in the small intestine supports the notion that naturally occurring human intestinal defensin is an effective arm of mucosal host defense. Its effectiveness may be maximal in the confined environment of the intestinal crypt, where Paneth cell secretions are likely to be concentrated. The vulnerable mitotic cells that continually repopulate the intestinal epithelial surface are located adjacent to the Paneth cells and may be protected by them against microbial invasion and parasitization. Paneth cell lysozyme and phospholipase A_2 (25) could potentiate defensins, and these interactions require further investigation.

In the presence of salt and at low pH, rHD-5 activity against *S. typhimurium* was much more impaired, perhaps reflecting the higher pathogenicity and virulence of this microorganism compared to those of *L. monocytogenes*. Nevertheless, rHD-5 was more bactericidal towards *S. typhimurium* than has been reported for murine intestinal defensins, cryptdins. In humans, large doses of *S. typhimurium* are needed to cause illness and, in contrast to disease in mice, the disease in humans is in general limited to the gastrointestinal tract. The resistance of wild-type *S. typhimurium* to cryptdin has led to suggestions that it is the cause of the virulence of this bacterium in mice (16, 51). It remains to be determined whether host specificity and pathogenicity of virulent microorganisms are codetermined by the specific antimicrobial spectrum of host antibiotic peptides.

ACKNOWLEDGMENTS

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