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 A₂ (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. Cystatins, the mouse intestinal defensins, have been isolated and proven to be active against Listeria monocytogenes, Escherichia coli, and Candida albicans. In contrast to cryptdins, the mouse intestinal defensins, HD-5 is active against both mouse-virulent wild-type Salmonella typhimurium and its isogenic, mouse-avirulent phoP mutant. In the presence of salt, HD-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.
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 $1.25 \times 10^6$ 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,
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 no 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 activity 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 log10 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 was only partially cleaved at the high concentration of the enzyme. In the presence of trypsin, the activity of rHD-5 against 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.
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 organism that are less affected by salt. Salt-resistant activity of rHD-5 was active at physiological salt concentrations whose viral envelopes are derived from 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 expect 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
The specific antimicrobial spectrum of host antibiotic peptides. Pathogenicity of virulent microorganisms are codetermined by it remains to be determined whether host specificity and it is the cause of the virulence of this bacterium in mice (16, wild-type general limited to the gastrointestinal tract. The resistance of in contrast to disease in mice, the disease in humans is in large doses of reported for murine intestinal defensins, cryptdins. In humans, was more bactericidal towards S. typhimurium was much more impaired, perhaps reflecting higher pathogenicity and virulence of this microorganism than has been than has been 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 small intestine crypt, where Paneth cell secretions are likely to be concentrated. The vulnerable mitotic cells that continually re-populate 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 A₂ (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 contributions to the purification of proHD-5, proHD-5Met, and rHD-5. This work was supported in part by the Public Health Service-National Institutes of Health grants HL-35640 and HL-46809.

ACKNOWLEDGMENTS
We thank Sylvia S. Harwig, Leonie Tan, and Hye Jin Yang for their suggestions to the purification of proHD-5, proHD-5Met, and rHD-5.