INFECTION AND IMMUNITY, Dec. 1994, p. 5397–5403
0019-9567/94/$04.00+0

Vol. 62, No. 12

Killing of *Giardia lamblia* by Cryptdins and Cationic Neutrophil Peptides†

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Received 23 June 1994/Returned for modification 16 August 1994/Accepted 16 September 1994

Antimicrobial polypeptides such as the defensins kill a wide range of organisms, including bacteria, fungi, viruses, and tumor cells. Because of the recent finding that intestinal defensins, also known as cryptdins, are synthesized by the Paneth cells of the small intestinal crypts and released into the lumen, we asked whether defensins and other small cationic antimicrobial peptides could kill the trophozoites of *Giardia lamblia*, which colonize the small intestine. Four mouse cryptdins, two neutrophil defensins (HNP-1 [human] and NP-2 [rabbit]), and the unique tryptophan-rich bovine neutrophil polypeptide indolicidin each had some antigiardial activity against trophozoites in vitro. Cryptdins 2 and 3, indolicidin, and NP-2 each reduced viability by more than 3 log units in 2 h, and killing by all peptides was dose and time dependent. Exposure of trophozoites to peptides frequently resulted in cell aggregation and dramatic changes in morphology. The mechanism of binding and lysis appeared to involve charge interactions, since 150 mM NaCl as well as millimolar levels of Ca^{2+} and Mg^{2+} inhibited killing by most of the peptides. Our studies show that *G. lamblia* is sensitive to defensins and indolicidin and suggest that these small polypeptides could play a role in nonimmune host defenses.

Giardia lamblia is the most common cause of protozoan disease of the human small intestine (3). Trophozoites are found in the lumen and can penetrate the mucus blanket and adhere to epithelial cells (8). Giardiasis has a broad clinical spectrum. Asymptomatic carriage is common, but the presentation can range from mild, self-limiting gastrointestinal upset to prolonged and debilitating diarrhea and malabsorption. In infants and young children, infection can result in failure to thrive or in growth retardation (2). Little is known about the factors which underlie this variability.

Although many immune and nonimmune mechanisms have been proposed as defenses against *Giardia* infection, the relative roles of specific and general host defenses, especially in the intestinal lumen, are poorly understood (4). The production of cationic peptides with antimicrobial activity is used as a nonimmune defense mechanism by a wide range of phylogenetically diverse organisms. One important class of such peptides in mammalian species is the defensins, which are small cysteine-rich cationic proteins characterized by nine highly conserved amino acids, including six cysteine residues (6, 9, 27, 33) (Fig. 1). They are present in rabbit alveolar macrophages and in human, rabbit, guinea pig, and rat neutrophils. Defensins can kill both gram-negative and gram-positive bacteria, acid-fast bacteria, fungi, enveloped viruses, and mammalian tumor cells (6, 9, 19, 24, 25, 35). The recent discovery of defensin molecules in the murine small intestine (6, 33) is of potential relevance to *Giardia* infection. Six enteric defensins, termed cryptdins, have been isolated from Paneth cells of small intestinal crypts and from the murine intestinal lumen, where they could come in contact with *Giardia* trophozoites.

Other antimicrobial peptides, differing from defensins in composition and structure, have also been identified in mammalian neutrophil granules. Like the defensins, indolicidin isolated from bovine granulocytes is a small cationic peptide, but the mature peptide has only 13 amino acids, contains no cysteine residues, and possesses a C-terminal amide. Named for its unusual abundance of tryptophan (38%), indolicidin is a structurally unique antimicrobial peptide (34).

In this paper, we report the sensitivity of *G. lamblia* to myeloid and enteric defensins and indolicidin and assess the potential role of these peptides in resistance to giardiasis.

MATERIALS AND METHODS

Cultivation of *G. lamblia*. Trophozoites of the C6 clone of *G. lamblia* WB (ATCC 30957) were grown to late logarithmic phase in TYI-S-33 medium (5) with bovine bile (18) but without added vitamins, iron, or antibiotics (12). Free-swimming trophozoites were discarded, and tubes with attached trophozoites were refilled with warm Dulbecco's phosphate-buffered saline (PBS). Trophozoites were detached by being chilled on ice for 10 min, harvested by centrifugation, and resuspended at 2×10^7/ml in 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5) with 9% (isotonic) sucrose. The use of this assay buffer permitted direct comparison with previously published reports on defensin activity in other systems (see, e.g., references 9 and 34). In some experiments, 4.5% (hypotonic) sucrose was substituted with no detectable difference in killing. Trophozoites were kept on ice for up to 2 h with no loss of viability. While the ionic composition of the small intestinal microenvironment is unknown, we did evaluate the effect of changes in the ionic

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† Publication 11 of the NCDDG-OI program, cooperative agreement UO1-AI-31696.

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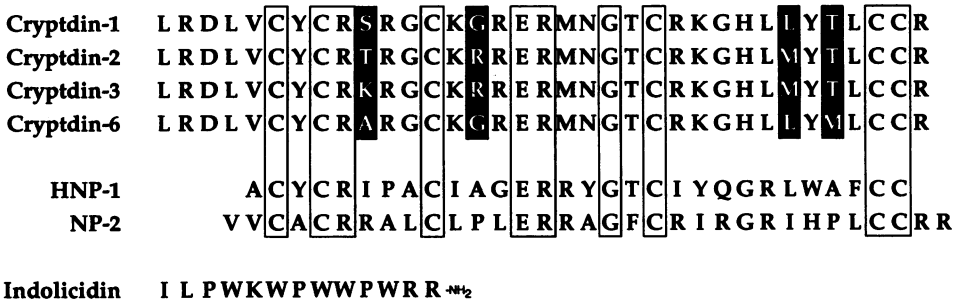

FIG. 1. Sequences of cryptdins 1, 2, 3, and 6 (33), a human neutrophil peptide (HNP-1) and a rabbit neutrophil peptide (NP-2) (9), and bovine indolicidin (34) and their relative alignment. Open boxes indicate the myeloid defensin conserved sequence motif. Black boxes indicate the amino acids which vary among the cryptdins.

composition of the buffer, particularly changes of Ca^{2+} and Mg^{2+} concentration, as described in Results. Unless otherwise specified, all chemical reagents were obtained from Sigma (St. Louis, Mo.).

Cysts were generated in vitro as described previously (11). Encysting cells (66 h) were harvested and resuspended in double-distilled water for 20 min at room temperature to lyse trophozoites and incomplete cysts. The remaining cysts were washed five times with water and stored overnight at 4°C. For dye uptake studies, mature cysts were enriched by centrifugation through a self-forming gradient of initially 27% Percoll (Pharmacia, Piscataway, N.J.) in PBS for 20 min at 10,000 × *g*, collected from the bottom of the gradient, and washed twice with HEPES-sucrose.

Antimicrobial peptides. Mouse cryptdins 1, 2, 3, and 6 (33), two neutrophil-derived defensins (rabbit NP-2 and human HNP-1 [9]), and bovine indolicidin (34) (Fig. 1) were purified to homogeneity as assessed by reverse-phase high-pressure liquid chromatography, acid-urea polyacrylamide gel electrophoresis, and amino acid analysis, as previously described (33). Stock solutions were prepared in 0.01% acetic acid and stored at −70°C until use. The maximum addition of 0.01% acetic acid was used as a control for each experiment.

Trypan blue assay of killing. In initial experiments, trophozoite killing was directly determined as loss of flagellar movement and membrane integrity as viewed under high-magnification (×400) Nomarski microscopy. In order to rapidly count larger numbers of cells, we determined that a trypan blue uptake assay correlated with direct observation both by comparison with parallel samples and by high-magnification microscopy of trypan blue-stained cells. Trophozoites or cysts were incubated with peptide in specified solutions for 2 h at 37°C. After incubation, cells were chilled for 5 min to detach any adherent cells, incubated on ice with an equal volume of 0.4% trypan blue (Gibco-BRL, Gaithersburg, Md.), and loaded into a hemocytometer for determination of numbers of live and dead cells. Unless otherwise stated, numbers represent the mean and standard deviation of three determinations. For logarithmic plots, 0% viable cells (100% killing) was plotted at the limit of detection for each experiment, ca. 1/500.

Excystation assay of cyst killing. Water-resistant cysts were preincubated with either 10 μg of cryptdin 2 per ml or 50 μg of indolicidin per ml in isotonic HEPES-sucrose for 1 h at 37°C, and then free peptide was removed by washing cysts with distilled water. Treated and control cysts were then excysted by our standard procedure without additional peptide, and emerging trophozoites were enumerated as described previously (1).

RESULTS

Killing of trophozoites by antimicrobial peptides. Initial experiments were performed to determine the susceptibility of *Giardia* trophozoites to neutrophil defensins, cryptdins, and indolicidin. While all of the peptides killed *G. lamblia*, their potencies varied dramatically. NP-2 and indolicidin reduced viability by nearly 3 log units (>99%) at concentrations of 100 and 50 μg/ml, respectively (Fig. 2A). In contrast, HNP-1, another neutrophil defensin, killed only 95% of trophozoites at the high concentrations of 200 and 400 μg/ml (Fig. 2A).

Even among the closely related cryptdins, the extent of killing varied considerably. Cryptdins 2 and 3 were the most effective peptides tested, killing by nearly 3 log units at a concentration of 20 μg/ml (Fig. 2B). In comparison, cryptdins 1 and 6 killed fewer than 75% of the trophozoites at the highest concentration tested. Killing was time dependent, and the differences in cryptdin potencies were observed throughout the 2-h assay period (Fig. 3).

Since defensins are believed to kill microbes by formation of pores in the target cell membrane, we tested the same concentrations of peptides in a hypotonic assay buffer (4.5% sucrose) in an effort to enhance killing of trophozoites. No measurable increase in either the rate or extent of killing by any of the tested peptides was seen (not shown). Killing was not observed if parasites were incubated at 4 instead of 37°C (not shown).

Morphologic effects of antimicrobial peptides. During the killing assays, particularly under hypotonic conditions, we frequently observed adherence of cells to each other, resulting in large aggregates (Fig. 4B and C). Although some clumping was observed in incubations with each of the cationic peptides tested, this aggregation appeared to be unrelated to the killing process. In each case the percentage of live trophozoites was the same for aggregated and dispersed populations, about 7% for 50 μg of NP-2 per ml (Fig. 4B) and 31% for 100 μg of HNP-1 per ml (Fig. 4C). Moreover, incubation under isotonic conditions substantially reduced or, in some cases, eliminated cell clumping (not shown) without any effect on killing. The rapid and severe distortion of trophozoites by indolicidin was unique among the peptides tested (Fig. 4D). In contrast to the aggregation induced by the defensins, this distortion correlated with trypan blue uptake and loss of flagellar movement, possibly reflecting the direct cytocidal effect of this peptide.

Effect of ion composition on trophozoite killing. Changes in the ionic composition of the assay buffer can alter the effectiveness of defensins. In particular, Ca^{2+} and sometimes Mg^{2+} can interfere with killing by defensins (20). We found that killing of *G. lamblia* by indolicidin was markedly inhibited by

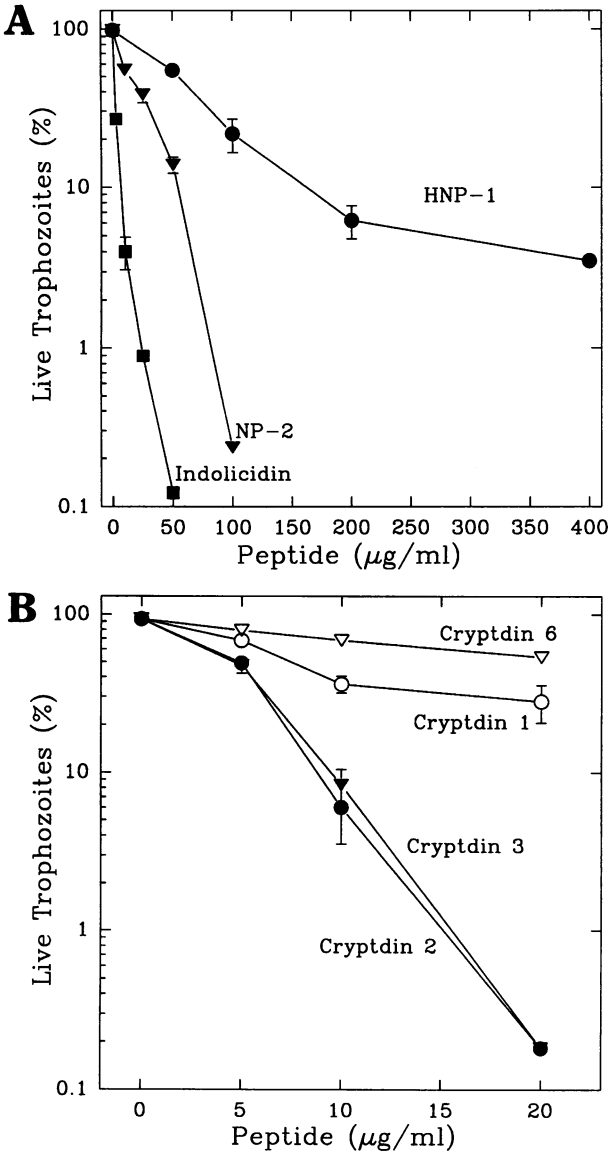

FIG. 2. Killing of *G. lamblia* trophozoites by antimicrobial peptides. Trophozoites were incubated with various concentrations of peptides for 2 h at 37°C, and trophozoite viability was determined by trypan blue exclusion. (A) Neutrophil cationic peptides indolicidin, NP-2, and HNP-1; (B) cryptdins 1, 2, 3, and 6.

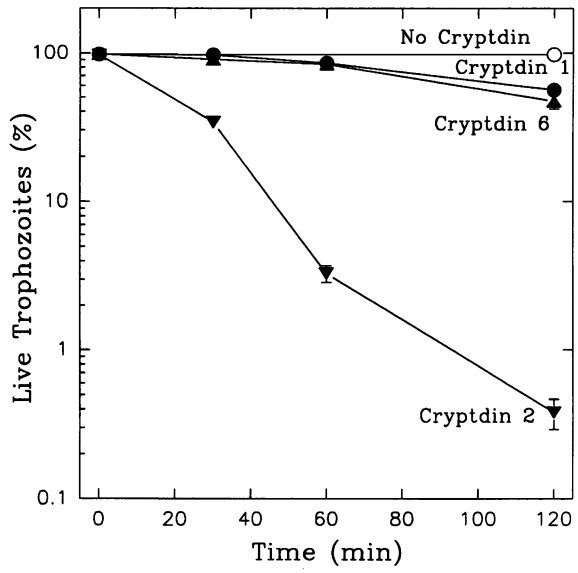

FIG. 3. Rate of killing at 37°C of *Giardia* trophozoites by 20 μg of cryptdin 1, 2, or 6 per ml.

low concentrations of Ca^{2+} but not Mg^{2+} (Fig. 5A). In contrast, both cations abrogated the action of cryptdin 2 (Fig. 5B) and the other neutrophil defensins (not shown). Although monovalent cations such as sodium could also inhibit killing by cryptdin 2 (Fig. 6) and other defensins (not shown), higher concentrations were required for equal inhibition. Indolicidin was active even in PBS (150 mM NaCl) but only at concentrations 5- to 10-fold higher than required in HEPES-sucrose (not shown).

Effect of peptides on viability of *G. lamblia* cysts. Cryptdins 2 and 3, indolicidin, and NP-2 decreased cyst viability as detected by trypan blue by 34 to 66% (Fig. 7). The killing of cysts was statistically significant ($P < 0.01$ for the highest concentrations of each peptide); however, cysts were much more resistant than trophozoites to the action of the most potent peptides (Fig. 7). Moreover, the rate of killing was not linear with time. Instead, there was an initial rapid loss of viability followed by a more gradual rate of death (not shown). We postulated that the initial rapid killing might be due primarily to the effect of peptides on immature cysts and that functional, mature cysts might be more resistant to peptide activity.

Effect of peptides on excystation. Such killing of immature cysts could lead to apparently high potencies of the peptides against cysts. Therefore, we tested preincubation of cysts with indolicidin and cryptdin 2 for blocking excystation, the primary function of mature *Giardia* cysts. Cryptdin 2, at 20 μg/ml, decreased excystation, indicating that it can affect fully developed cysts, but the 64.0% inhibition remains well below the three-orders-of-magnitude killing of trophozoites. In contrast, 25 μg of indolicidin per ml caused no detectable decrease in excystation, suggesting that mature cysts are totally resistant to this concentration of peptide (data not shown).

DISCUSSION

Defensins and other antimicrobial peptides have toxic activity against a wide range of organisms, including gram-negative and gram-positive bacteria, acid-fast bacteria, fungi, enveloped viruses, and mammalian tumor cells (6, 9, 19, 21, 24, 25, 35). The first defensins to be characterized were peptides expressed by neutrophils, cells thought to play a minimal role in most *Giardia* infections. However, the recent discovery that specific defensins (cryptdins) are expressed by small intestinal cells has generated new interest in the role of these peptides in mucosal immunity.

Cryptdins are synthesized constitutively by Paneth cells, which are nonmotile granulocytes located in the base of the crypts of Lieberkuhn in the small intestine (26). Cryptdins contain the conserved sequence motif which characterizes all defensins (Fig. 1, open boxes), including the six disulfide-linked cysteines which stabilize the peptides (13, 30). Although the absolute amounts of cryptdins are unknown, immunoperoxidase staining, as well as analysis of intestinal lavage fluid, has demonstrated that in mice cryptdins are constitutively

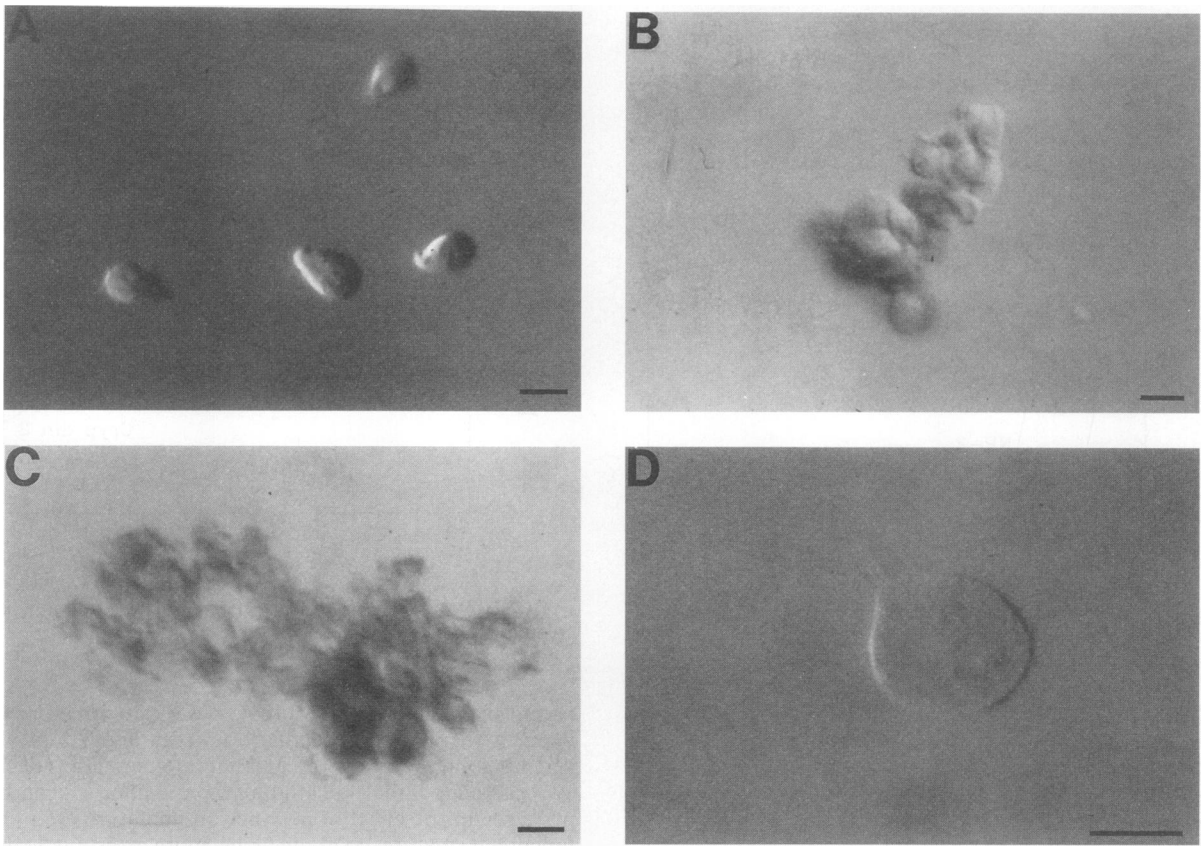

FIG. 4. Effect of NP-2, HNP-1, and indolicidin on trophozoites. Trophozoites were incubated for 2 h at 37°C in 25 mM HEPES (pH 7.5) plus 4.5% sucrose with no peptide (A), 100 μg of HNP-1 per ml (31.2 ± 9.1% viability) (B), 50 μg of NP-2 per ml (6.7 ± 2.6% viability) (C), or 5 μg of indolicidin per ml (59.6 ± 9.4% viability) (D). Trypan blue was added to 0.2% before viewing. Bars, 10 μm.

secreted into the crypts, diffuse into the intestinal lumen, and coat the mucosal surface (33). Thus, *Giardia* trophozoites colonizing the small intestine would encounter cryptdins at increasing concentrations in the lumen and during penetration of the mucus layer and entry into the crypts. If *Giardia* trophozoites are susceptible to killing by luminal cryptdins in any of these varied physiologic environments, the peptides may play a significant role in mucosal resistance to this protozoan pathogen.

Each of the antimicrobial peptides that we tested had some concentration- and time-dependent giardicidal activity, but their potencies varied widely (Fig. 2). The human neutrophil defensin HNP-1 was the least active of the peptides tested, while NP-2, a rabbit neutrophil defensin, and indolicidin were both highly effective at relatively low peptide doses (Fig. 2). However, the most potent of the peptides we tested were two mouse cryptdins, cryptdins 2 and 3. These peptides caused a reduction of nearly 3 log units in viability of trophozoites at 20 μg/ml. In contrast, cryptdins 1 and 6 had substantially less effect at concentrations of up to 20 μg/ml. The giardicidal concentrations of defensins were only slightly higher than bactericidal concentrations (e.g., a reduction of 4 log units in *Escherichia coli* CFU is observed with 10 μg of indolicidin per ml [34]) and substantially less than tumorcidal concentrations (e.g., a reduction of < 2 log units in K562 cells is observed with 100 μg of NP-2 per ml [24]) under similar conditions. Differences in killing among different cryptdins and other defensins have been observed previously in studies on avirulent *Salmonella typhimurium* (6). It is interesting, however, that the ranking of potencies with *S. typhimurium*, i.e., NP-2 >> cryptdin 1 >> cryptdin 2, was markedly different from that with *G. lamblia*, i.e., cryptdin 2 >> NP-2 >> cryptdin 1.

The difference in potencies of the cryptdins is intriguing because of their extensive sequence similarities. The amino acid sequences of the four cryptdins tested differ in only four positions, and the substitutions are mainly conservative (Fig. 1, black boxes). Only the substitution of an arginine residue for glycine 15 appears to correlate with increased cryptdin activity against *G. lamblia*.

The crystal (13) or solution (29, 30) structures of four different myeloid defensins are known. They all possess a similar peptide fold with beta-sheet predominance and an amphipathic surface topology. These amphipathic molecules permeabilize target cell membranes by a mechanism thought to involve the formation of transmembrane pores. Defensin peptides induced voltage-gated channels in planar lipid bilayers, and the peptide concentration dependence suggested that the functional pore required a multimeric peptide assembly (17, 23). If this is the mechanism for killing of *G. lamblia*, it is possible that the greater potencies of cryptdins 2 and 3 (compared with cryptdins 1 and 6) are related to their ability to assemble in the membrane of the trophozoite. Studies are currently under way to determine the stoichiometry of assembly for each of the cryptdins characterized thus far.

The differing giardicidal potencies of these peptides could also be a function of their relative efficiencies of binding to the trophozoite surface. Binding of the peptides to the surface of *Candida albicans* or *E. coli* preceded killing, and the antimi-

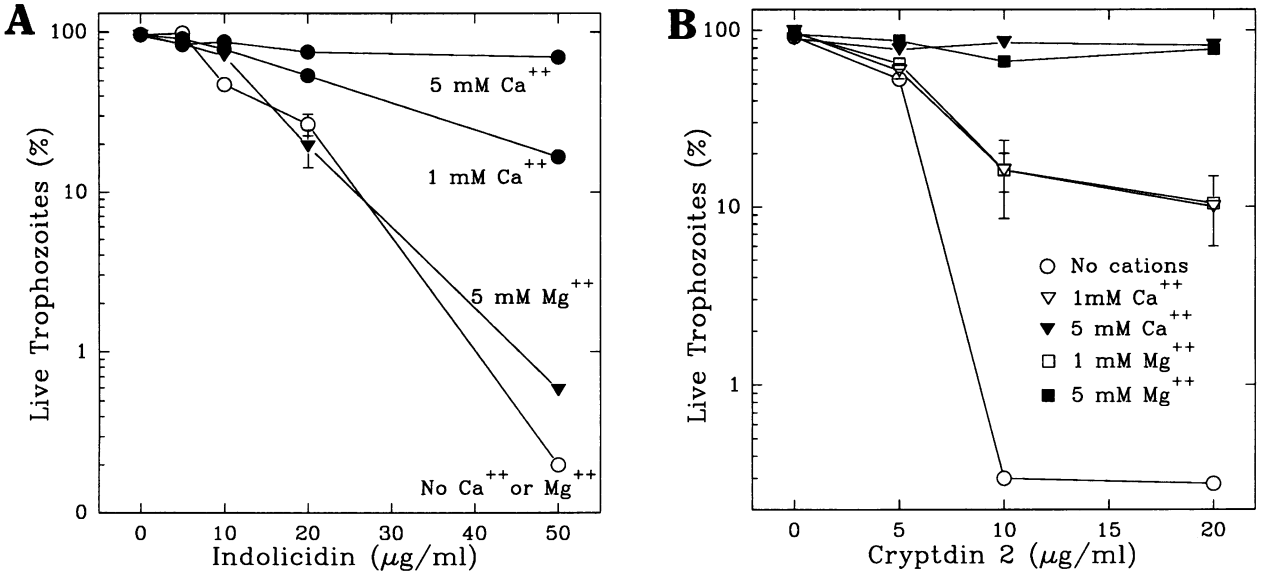

FIG. 5. Effect of divalent cations on killing of *Giardia* trophozoites by indolicidin (A) or cryptdin 2 (B).

crobial potency was related to the amount of peptide bound. Moreover, these initial peptide-target interactions were markedly inhibited by divalent and, to a lesser extent, monovalent cations (20, 22). We demonstrated a similar effect in the present study. The addition of 5 mM Ca^{2+} or Mg^{2+} dramatically reduced cryptdin 2 giardicidal activity (Fig. 5), whereas the addition of 10 or 20 mM NaCl was less inhibitory (Fig. 6). The consequences of this inhibition by cations are difficult to predict. In particular, little is known of the ionic composition of the mucus layer and within the crypts of the small intestine, the microenvironment where cryptdin concentrations are likely to be the highest and the region where the critical interaction of trophozoites and intestinal cells occurs. Moreover, the composition of the lumen may be quite variable.

The effect of metal ions on the giardicidal activity of indolicidin was distinctly different from their effect on the defensins. Specifically, Ca^{2+} readily inhibited activity, but comparable concentrations of Mg^{2+} had no effect. Because the structure of this antimicrobial peptide is quite different from that of defensins, it is not surprising that factors which modulate defensin function do not necessarily apply to this small linear molecule. In contrast to the inhibitory effects of high ionic strength, adjustment of the osmotic strength of the assay medium with sucrose had no apparent effect on killing of trophozoites by defensins or indolicidin.

We also observed that antimicrobial peptides could have direct morphologic effects on trophozoites, particularly at low osmotic strength. Addition of defensins was followed by the rapid formation of cellular aggregates (Fig. 4B and C), a finding not reported for bacteria or fungi. However, the percentages of viable trophozoites were similar for aggregated and dispersed cells, suggesting that the cell clumping was unrelated to killing. In contrast, the deformation of trophozoites by indolicidin correlated with killing and may be a clue to the mechanism of cell killing by that peptide.

Giardia cysts were markedly less susceptible than trophozoites to killing by antimicrobial peptides. Moreover, mature cysts capable of excysting in response to appropriate environmental stimuli were even more resistant to peptide action. It is likely that the heavily cross-linked cyst wall is responsible for much of the resistance, possibly by concealing or destroying the normal binding sites of the peptides or by blocking penetration of the plasma membrane by peptides. The differences in peptide potencies against trophozoites or cysts support the hypothesis that the effect of cationic peptides is more likely to be on disease (trophozoite stage) than on infection (cyst stage).

Our results suggest that intestinal defensins could affect the course of infection by *G. lamblia*, a hypothesis supported by other indirect evidence. Suckling mice (3 days old) are readily infected by *G. lamblia* trophozoites (14). By 13 to 17 days of age the trophozoite population begins to decline, and between 17 and 20 days of age the infection rapidly drops below the level of detection. Immunocompetent adult mice are not susceptible to infection with *G. lamblia* (14). Cryptdin mRNAs

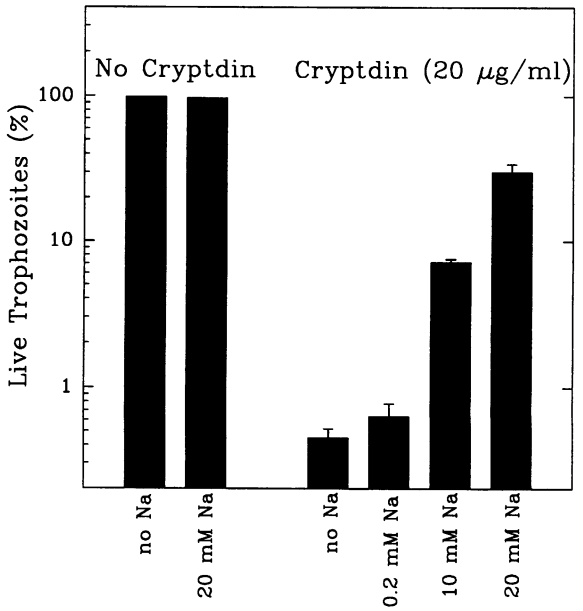

FIG. 6. Effect of $[Na^+]$ on killing of *Giardia* trophozoites by cryptdin 2.

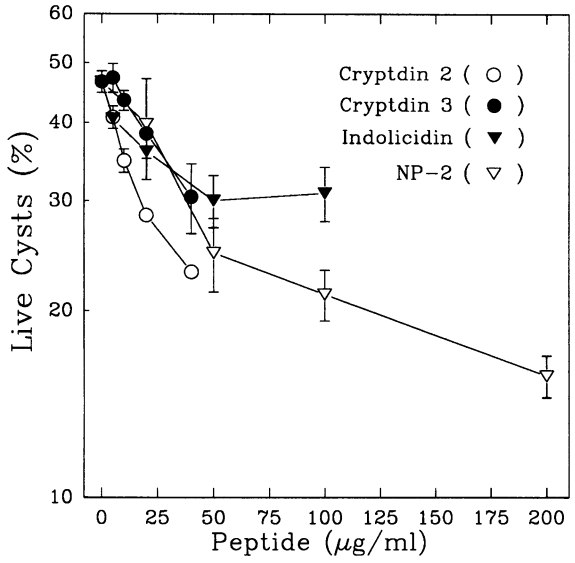

FIG. 7. Effect of antimicrobial peptides on *Giardia* cyst viability as determined by trypan blue exclusion. Percoll-purified cysts were incubated for 2 h at 37°C with various concentrations of peptides, as described in Materials and Methods. Note that the vertical scale covers only 1 log unit.

are first detected by in situ hybridization in 10-day-old mice, but in only about 10 to 20% of crypts (26). By 16 days, 100% of crypts synthesize cryptdins, and by 20 days, maximal levels of synthesis are detected. Increasing concentrations of cryptdins in the developing mouse intestine could be one of the factors involved in the developmental acquisition of resistance to *G. lamblia* in mice.

It is interesting that, at least in infections of mice with *Giardia muris*, large numbers of trophozoites may be found attached to the mucosa at the base of the villi, near or in the crypts (28). While one report has suggested a possible phagocytic role of Paneth cells (7), they are known to have a secretory function in the small bowel (10, 31, 32). We suggest that the secretion of cryptdins and other Paneth cell products may provide an important component of local host defenses against giardial infection. The wide variability of symptoms in giardiasis might be partly explained by differences in defensin composition and secretion into the intestinal milieu. Furthermore, because even closely related peptides can have widely different toxic effects, slight allelic differences in defensins could have major effects on host susceptibility to *Giardia* spp. and other agents. Recently, two cDNA clones of putative human intestinal defensins have been sequenced (15, 16). As purified peptides become available, it will be interesting to test the effects of human cryptdins on *Giardia* viability. In addition, future studies on the regulation of synthesis and secretion of cryptdins may shed light on the host response to transient and chronic microbial colonization of the small intestine by *Giardia* spp. and other pathogens.

ACKNOWLEDGMENTS

We thank Heather Shepherd for excellent technical assistance.

This work was supported by NIH grants AI-19863, AI-24285, AM-35108, HD-29275, AI-22931, and AI-31696.

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