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Giardicidal Activity of Lactoferrin and N-Terminal Peptides

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Human and bovine lactoferrins and their derived N-terminal peptides were giardicidal in vitro. Fe^{3+}, but not Fe^{2+}, protected trophozoites from both native lactoferrin and peptides, although the latter lack iron-binding sites. Other divalent metal ions protected only against native lactoferrin. Log-phase cells were more resistant to killing than stationary-phase cells. These studies suggest that lactoferrin, especially in the form of the N-terminal peptides, may be an important nonimmune component of host mucosal defenses against *Giardia lamblia*.

Both immune and nonimmune mechanisms may be important influences on the incidence, duration, or severity of giardiasis. Factors active in the upper small intestine may be produced by intestinal tissue, secreted into intestinal fluid, or ingested by breast-fed babies. The prevalence of infection appears lower in the first 6 months of life, increasing after weaning (10). In addition to containing secretory antibodies, human milk is a concentrated source of nonimmune antimicrobial factors such as lactoferrin (LF), which has been proposed to help protect babies from bacterial diarrhea (9). LF, which binds two ferric ions, is the main iron-binding protein of the mucosa, including the intestinal epithelium, and is also produced by neutrophils and lymphocytes (11, 15, 16, 18, 20). Thus, it is present at the site of giardial colonization.

LF has broad-spectrum antibacterial and antifungal activities and is considered an important component of host defenses at mucosal surfaces. Although its microbicidal action was originally attributed to withholding iron from susceptible organisms, LF causes more rapid killing than iron deprivation, and that killing is not necessarily prevented by iron (2–4, 13). Moreover, N-terminal antimicrobial peptides (lactoferricin) (LFpep's) generated by gastric pepsin cleavage of human (residues 18 to 40 of the native molecule) and bovine (residues 17 to 41) LFs (HLF and BLF, respectively) have more potent bactericidal activities than the native proteins, although they lack the iron-binding sites (5–8, 22, 24). Therefore, we investigated LF and the corresponding LFpep fragments as potential nonimmune secretory defenses against *Giardia lamblia.*

HLF was obtained from Sigma Chemical Company (St. Louis, Mo.), and BLF and BLFpep were generous gifts of the Morinaga Milk Company, Zama City, Japan. HLFpep was synthesized by Quality Controlled Biochemicals, Inc. (Hopkinton, Mass.) by the method of Bellamy et al. (6). Purity of both peptides was demonstrated by reverse-phase high-performance liquid chromatography (6) (Quality Controlled Biochemicals).

Killing activity was assayed as described previously for defensins (1) in a 50-μl volume in siliconized Microfuge tubes, except that the final trophozoite concentration was 10^7/ml.

Giardicidal activities of LF and LFpep. On a molar basis, BLFpep had the most potent giardicidal activity, followed by HLFpep, BLF, and HLF (Fig. 1). The 50% lethal doses (LD_{50}s) were as follows: BLFpep, 2.6 μM (8 μg/ml); HLFpep, 5.4 μM (16 μg/ml); BLF, 15.0 μM (1.2 mg/ml); HLF, 18.7 μM (1.5 mg/ml). In subsequent experiments, BLFpep at 12 μg/ml, HLFpep at 24 μg/ml, BLF at 2.0 mg/ml, and HLF at 2.5 mg/ml were used to produce equivalent killing, decreasing trophozoite viability from ≥95% to the following levels: BLFpep, 21.9% ± 1.3%; HLFpep, 24.6% ± 3.1%; BLF, 20.6% ± 3.6%; HLF, 25.4% ± 2.5%. Kinetic analyses showed no significant lag before the initiation of killing, which continued throughout the 2-h incubation with a half time of ~60 min.

The LF concentration in human colostrum is approximately 15 mg/ml and in human milk is at least 1 mg/ml (16, 18). Moreover, each full contraction of the gallbladder releases 10 to 30 mg of LF into the duodenal lumen (11). Thus, LF and LFpep were giardicidal at physiologic concentrations and may interact with both luminal and attached trophozoites. To our knowledge, this is the first demonstration of killing of a human parasite by LF or LFpep. While all LF forms tested were giardicidal, the cleaved peptides are substantially more potent in killing *G. lamblia*, certain bacteria (5, 6), and candida (7). In recent studies (22a), we found that both human and bovine LFs and LFpep's bound to the surface of live trophozoites in the cold, as demonstrated by indirect immunofluorescence with antibodies against HLF and BLF, which also reacted with the respective peptides. Moreover, active BLFpep was generated in quantity during passage through the rat stomach (22). Under different conditions, LF stimulated uptake of iron by and activity of certain enzymes in *Trichomonas vaginalis* (17).

Interestingly, giardial trophozoites harvested from very-low-density cultures were highly resistant to LF, and somewhat resistant to LFpep, compared with trophozoites from late-log-phase cultures (produced by varying the inoculum during 72 h of incubation). Trophozoites from early-stationary-phase cultures were strikingly more susceptible to killing. Importantly, only attached (active) trophozoites were used in all studies, and the viability of controls was equivalent in all growth phases (Table 1). In contrast, certain bacteria were more resistant to LF with increasing culture age (4).

Effects of metal ions and environmental conditions. As was true of many bacteria (24), ferric iron as $Fe_2(SO_4)_3$ (Table 2) or $FeCl_3$ (not shown) protected trophozoites, with 50% protection at 0.2 to 0.3 mM, although ferrous sulfate did not protect at 5 mM. Protection by iron against LFpep was particularly interesting, as the peptides lack the two iron-binding sites of native LF. $MgCl_2$, $CaCl_2$, and $CoCl_2$ (5 mM) also strongly decreased killing by native LF (Table 2); however, unlike ferric iron, these metal ions did not protect against

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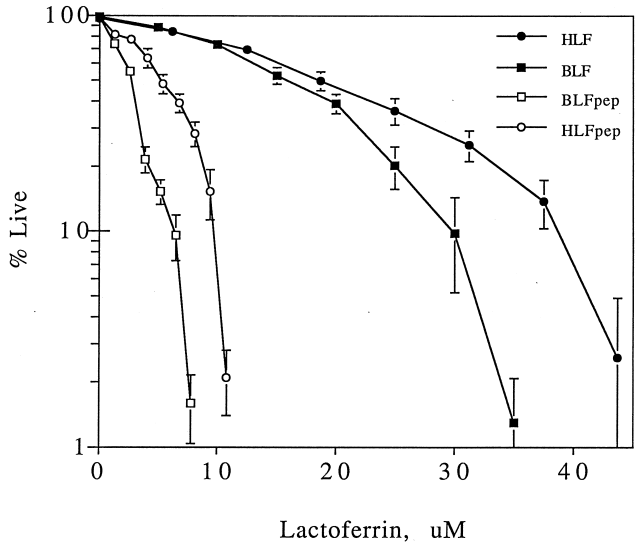

FIG. 1. Effect of concentration on giardicidal activities of HLF, HLFpep, BLF, and BLFpep. Trophozoites were incubated with the indicated concentrations of LF or LFpep for 2 h at 37°C, and viability was determined by trypan blue exclusion, which correlated well with mobility and morphology. Bars, standard deviations.

LFpep (Table 2). It is unclear how either ferric iron or these divalent cations interfered with giardicidal activity. Under identical conditions, 5 mM $CuSO_4$ or $CrCl_3$ or 50 mM NaCl, Na_2SO_3, or KH_2PO_4 did not protect trophozoites against LF or LFpep.

Intestinal factors and physiologic conditions, including small intestine pH (from 6.0 to 8.5), bile salts (10 mM taurocholate or glycocholate), and changing the osmolarity of the assay buffer from isotonic (9% sucrose) to hypotonic (4.5% sucrose) or hypertonic (18% sucrose), had no effect on killing of *G. lamblia* by LF or LFpep. In addition, neither human transferrin nor lysosome (4 mg/ml) had giardicidal activity or any additive or protective effect at concentrations equivalent to the LD_{50}s of LF and LFpep.

LF, at physiologic concentrations, killed trophozoites under our in vitro assay conditions; however, metal ions present in the small intestine could be protective. Ca^{2+} and Mg^{2+} block the activity of native LF, as well as other well-characterized

TABLE 1. Effect of *G. lamblia* growth phase on susceptibility to LF and N-terminal peptides

LF	% Survival in the following phase[a]:		
	Very early log	Late log	Stationary
None	97.3 (10.0)	99.3 (9.6)	98.0 (12.8)
HLF	90.7 (10.5)[b]	22.0 (12.0)[c]	2.2 (1.7)[b,c]
HLFpep	58.4 (9.4)[b,c]	21.6 (2.3)[c]	7.5 (1.0)[b,c]
BLF	89.2 (12.4)[b]	24.2 (4.5)[c]	1.3 (1.7)[b,c]
BLFpep	46.0 (4.3)[b,c]	23.7 (3.0)[c]	1.3 (1.1)[b,c]

[a] The data are means (with standard deviations in parentheses) for at least three experiments done on three separate occasions. Initial trophozoite concentrations per milliliter: very early log phase, 2×10^4; late log phase, 10^6; stationary phase, 2.7×10^6. Trophozoites at all stages were adjusted to a final concentration of 10^7/ml for the assay.

[b] Significantly different from the corresponding value for late-log-phase cells ($P < 0.01$ [one-tailed Student's t test]).

[c] Significantly different from the corresponding value for the no-LF control ($P < 0.01$ [one-tailed Student's t test]).

TABLE 2. Effects of metal ions on killing by LF and N-terminal peptides

LF	% Survival with the following metal added[a]:				
	None	$Fe_2(SO_4)_3$	$MgCl_2$	$CaCl_2$	$CoCl_2$
None	95.9 (1.0)	96.2 (1.7)	95.6 (2.3)	95.7 (1.8)	97.3 (5.4)
HLF	24.6 (5.5)[b]	81.9 (5.1)[c]	93.9 (4.7)[c]	91.0 (6.8)[c]	92.7 (4.0)[c]
HLFpep	21.3 (4.2)[b]	70.5 (3.5)[c]	23.7 (4.1)[b]	24.3 (1.3)[b]	22.7 (3.0)[b]
BLF	20.1 (5.0)[b]	70.5 (9.5)[c]	86.2 (7.9)[c]	86.7 (3.5)[c]	85.8 (4.9)[c]
BLFpep	24.4 (0.5)[b]	71.9 (16.4)[c]	21.9 (0.3)[b]	22.1 (1.0)[b]	23.5 (0.2)[b]

[a] 5 mM. The data are means (with standard deviations in parentheses) for at least three experiments done on three separate occasions.

[b] Significantly different from the corresponding value for the no-LF control ($P < 0.01$ [one-tailed Student's t test]).

[c] Significantly different from the corresponding value for the no-metal control ($P < 0.01$ [one-tailed Student's t test]).

polycationic membrane-active agents, including defensins (1, 12, 14, 21, 23). In contrast, killing by LFpep was not inhibited by any metal ion tested, except for Fe^{3+}. Since LF in human milk is only 3 to 5% iron saturated and the intestinal lumen has little free iron, it is likely that LFpep would remain active within the human small intestine.

As with other nonimmune giardicidal factors including cryptdins (1) and bile salt-stimulated lipase (19), the net effect of LF or LFpep on trophozoites may depend on the precise microenvironment and local concentrations of possible protective factors. This complexity of giardicidal versus protective conditions in the intestinal milieu may help explain the variability of giardial infection.

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