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Killing of Giardia lamblia Trophozoites by Normal Human Milk

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The clinical course of giardiasis is variable, and serum antibodies do not appear to be protective. We propose that natural factors either produced by intestinal tissue, transported into the intestine, or ingested (ie, by breast-fed babies) might promote resistance to this disease. Human milk is very rich in secretory IgA (S-IgA) antibodies, as well as nonspecific antibacterial factors (eg, lactoferrin, lysozyme).

Previous studies showed that Giardia lamblia trophozoites were killed by nonimmune human milk (NHM) in a time- and concentration-dependent manner. Removal of >99% of the S-IgA from NHM did not decrease its Giardia-cidal activity. Thus, the killing was not antibody dependent. This is the first demonstration of nonimmune antiparasitic defenses in human milk.

The present studies show that in the presence of NHM, trophozoites lost motility, swelled, and lysed. The Giardia-cidal activity (GCA) may be specific to human milk, since unheated cow's and goat's milk were virtually devoid of activity. Much, but not all, of the GCA was lost when NHM was heated or reacted with diisopropylfluorophosphate (DIFP), a specific esterase inhibitor. Activity of the major human milk lipase (BSL, bile salt-stimulated lipase, a fatty acid esterase) was lost after heat or DIFP treatment and was absent from cow's or goat's milk. The parasites were also killed by pure BSL. These studies suggest that BSL may be a heat-labile Giardia-cidal component of NHM.

Key words: parasite, bile salt-stimulated lipase

Giardia lamblia is a major cause of enteric disease throughout the world. This parasite is a binucleate flagellated protozoan. Although actively motile, it frequently attaches by its ventral adhesive disc to the intestinal epithelium in vivo or to the walls of culture vessels in vitro [1,2]. G lamblia trophozoites (like the Entamoeba and trichomonads) have no mitochondria and a limited tolerance of air, but consume oxygen [3,4]. This pathogen has only recently been grown axenically in complex media under reduced O_2 tension [5,6].

The symptoms of giardiasis vary from asymptomatic infections to debilitating diarrhea and malabsorption. Illness may resolve spontaneously within a few days or

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persist for years despite the presence of circulating or secretory antibodies. Giardiasis is especially common in children and may cause failure to thrive [7].

Little is known about human defenses against G lamblia. It is likely that both immune and nonimmune defenses influence the course and severity of the disease. In a murine model, infants were protected from G muris infection by milk from previously infected mice [8].

Infection occurs by ingestion of cysts, which are triggered to excyst by exposure to low gastric pH [9]. Trophozoites emerge and colonize in the upper small intestine, a complex and ever-changing environment. Duodenal fluid contains a variety of degradative enzymes and is subject to strong forces moving it "downstream." In aspirates or biopsy material, live trophozoites may be observed in the intestinal fluid, associated with mucus or adherent to the epithelium. Sickled, nonmotile (presumably dead) trophozoites and ghosts are also observed even in chronic infections [Gillin and Nash, unpublished observations].

For these reasons, we became interested in the possibility that natural or immune factors in the human gastrointestinal tract might mediate resistance to giardiasis. Such factors could either be produced by intestinal tissue, transported into the intestine or ingested (ie, by breast-fed babies). It is particularly relevant that G lamblia colonizes the upper small intestine, where it would be exposed to high concentrations of pancreatic enzymes and milk, in the case of breast-fed babies.

Human milk is very rich in secretory IgA (S-IgA) antibodies, as well as nonspecific antibacterial factors (eg, lactoferrin, lysozyme), which may also be present in intestinal secretions. Milk is a more uniform and easily available source of these factors.

Our original hypotheses were that secretory antibodies would be the protective factor and that they would protect by interfering with Giardia attachment, rather than by killing.

However, in earlier studies, we observed that G lamblia trophozoites were killed by normal human milk (NHM) in a time- and concentration-dependent manner. One hundred percent killing was observed in 5 h with 0.3% milk, in 2 h with 1% milk, or in less than 0.5 h with 3% milk. The kinetics of killing were nonlinear with respect to milk concentration as with 0.3% milk, a 3-h lag was followed by rapid killing [10]. Removal of >99.5% of the S-IgA from NHM did not decrease its Giardia-cidal activity at all. Thus, the killing was *not antibody dependent* [10].

The present studies show (1) that the Giardia-cidal activity (GCA) may be specific to human milk, as neither cow's nor goat's milk killed this parasite; (2) human milk has both heat-labile and heat-stable GCA; and (3) an unusual lipase, found in the milk of humans and gorillas, but not of lower mammals, may constitute a heat-labile lethal factor in milk.

MATERIALS AND METHODS

Parasites and Cultivation

Giardia lamblia strain WB, American Type Culture Collection (ATCC) No. 30957, isolated from the duodenal fluid of a patient with symptomatic giardiasis of 2.5-y duration [11], was used in most studies. Giardia strain PO (5,6 ATCC No. 30888) was used in early experiments. Results of control studies showed no differences in milk sensitivity between the strains or between cloned or uncloned strain WB

organisms or parasites grown in TYI-S-33 or TP-S-1 media [6]. Pure cultures were grown to late log phase in modified TYI-S-33 medium (trypticase, yeast extract, serum, and bile are the complex components of this medium [12, 13] with subculture twice weekly.

Milk Samples

Samples of mature human milk tested initially were the generous gift of Dr V. Ginsburg (National Institutes of Health). Other samples were obtained from women with no history of giardiasis or other intestinal disease. Fresh goat's milk was the generous gift of the ungulate unit of the NIH Animal Center and cow's milk of the University of Maryland Dairy. The cow's milk, goat's milk, and the first samples of human milk were centrifuged (9,800g, 40 min, 4°C) to remove cells and fat. Since this procedure did not affect the killing activity (see Results), it was discontinued. Milk samples were stored frozen in small volumes to avoid repeated freezing and thawing.

Bile Salt-Stimulated Lipase (BSL)

Early preparations of BSL from human skim milk were purified to apparent homogeneity by sequential chromatography on columns of concanavalin A-Sepharose 4B and cholate Sepharose [14]. Later, cholate Sepharose, Bio-Rex 5, and heparin-Sepharose were utilized [15]. These preparations had a single band upon sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, and a unique 22-residue amino-terminal sequence was determined.

BSL (EC 3.1.1.3.) is a carboxylesterase that hydrolyses both long and short chain fatty acids from either water soluble or insoluble substrates [16]. Enzyme activity was assayed by a tritrimetric method with emulsified tributyrin as substrate [17], or by a more sensitive spectrophotometric assay using p-nitrophenyl acetate [15]. Sodium taurocholate (2 mM) was used as activator with both substrates.

Other Reagents

Other reagents were purchased from Sigma Chemical Corp., unless otherwise specified, and were of the highest available purity.

Experimental Procedures

In log phase G lamblia cultures, >90% of the organisms are attached to the walls of the culture vessel [18]. Nonattached organisms, including any dead ones, were removed with the culture medium. The growth medium was replaced with protein-free maintenance medium (MM) [2] and parasites were detached by chilling for 10 min in an ice-water bath. The trophozoites were washed twice in this medium by centrifugation and suspended in the same medium or in TYI-S-33 medium lacking the serum and bile components [13]. Experiments were initiated by addition of 0.5 ml of parasite suspension to 1 dram (4 ml) screw-capped glass vials containing the milk or other test substances diluted in protein-free MM or in phosphate buffered saline (PBS), to a final volume of 1 ml. The reactions were incubated for 3 hr at 37°C, and parasite survival was determined by (1) direct microscopic assay and/or (2) colony assay [19]. The results obtained with these assays were equivalent. In many experiments, both assays were performed. The initial cell concentration in the first assay was 2×10^5/ml and in the colony assay $1–2 \times 10^4$/ml. Control experiments

with both assays showed that the percentage of parasites killed did not vary with parasite concentration within this range.

Direct microscopic assay. Experiments were terminated by the addition of 3 ml of complete growth medium containing serum, which arrested killing. The vials were then incubated overnight at 37°C. Surviving parasites attached to the vial and were counted in situ using an inverted microscope (Nikon). Killed organisms were not visible (see Results). Five random microscope fields containing 50 or more organisms or ten fields containing fewer, were counted.

Colony assay. The vials were chilled after addition of complete growth medium and mixed to create a uniform single-cell suspension. Duplicate cell samples were added to tubes of melted agarose growth medium. After the medium solidified, cultures were incubated for 4–7 days at 37°C. Individual surviving parasites grew into visible colonies (clones) and were counted [19].

At low milk concentrations (0.3–1%) there was some variation in survival from experiment to experiment; ie, at 1% milk, survival varied from 0% to 20%. This is probably a reflection of the complex, biphasic kinetics of killing [10], in which a small change in the duration of the lag prior to killing can result in a large change in the killing measured at a particular time and milk concentration. For this reason, all experiments were carried out at multiple milk concentrations and *absolute* data can be compared only *within* a given experiment.

RESULTS

In preliminary experiments, milk from seven normal women killed >80% of G lamblia in 3 h at a concentration of 1%, but <20% were killed at 0.1%. In the presence of lethal milk concentrations, trophozoites soon detached and became immotile and ghostlike with nuclei easily visible without staining. Some organisms became swollen and rounded (Fig. 1A). In contrast, control organisms retained normal morphology (half-pear-shaped, nuclei not visible), motility, and attachment to the vials (Fig. 1B). After overnight incubation, neither ghosts nor other remnants of the killed organisms were visible. This was the basis of the direct microscopic assay for survival.

In contrast to the potent Giardia-cidal activity (GCA) of normal human milk, unheated cow's or goat's milk had very little killing activity (Table I). Partial killing by cow's or goat's milk was observed only at much higher concentrations (~20%) than human milk (0.3–1%). In other experiments, little killing was observed with 50% cow's or goat's milk. In fact, stimulation of parasite survival (or multiplication) in the serum-free assay medium was observed with 1.7% or 4.15% cow's or goat's milk (Table I). This was also frequently observed with sublethal concentrations (0.1% or less) of human milk.

Since human milk is a complex biological fluid, attempts were made to characterize the GCA. First, milk was centrifuged (10°C, 30,590g, 30 min) to sediment cellular material and casein micelles and to separate the lipid-rich "cream" fraction. Although the top (fat) and bottom layers contained some skim milk, it was clear that both skim and whole milk, as well as the upper and lower fractions, had potent killing activity [Table II, A]. In addition, killing activity was not lost after extensive dialysis of NHM against PBS [Table II, B].

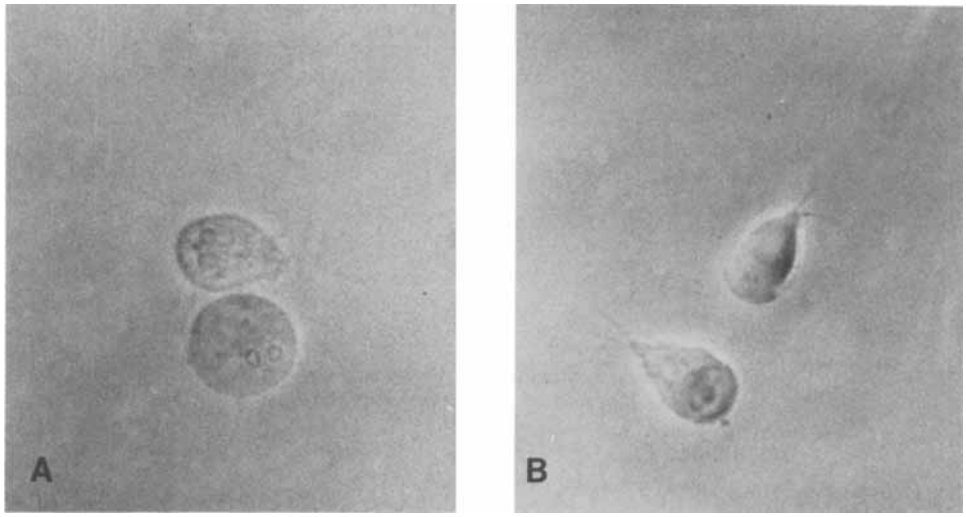

Fig. 1. Early effects of normal human milk on Giardia lamblia trophozoite morphology and motility. Parasites were exposed to 5% NHM (A) or buffer (controls, B) for 2 hs at 37°C. The reaction mixtures were then chilled for 10 min and centrifuged to concentrate the cells, which were suspended in complete growth medium and photographed with a Zeiss microscope under phase contrast. A. Two non-motile cells: flagella and nuclei are visible. B. Motile, active cells: posterior flagella are visible in both cells. In one cell the adhesive disc is visible anteriorly and the ventral groove with beating flagellum posteriorly. ×1,000.

TABLE I. Cow's Milk and Goat's Milk Have Little Giardia-cidal Activity

Milk concentration (% vol/vol)	Survival (% control)[a]		
	Human	Cow	Goat
1.7	0	124	152
4.15	0	131	128
22.7	0	58	31

[a]Survival was determined by colony assay. Controls lacking milk (1,592 colonies/ml) were considered 100%.

Most of the GCA was thermolabile, as no killing was observed with 1% (or less) NHM after heating at 70°C for 5 min (Fig. 2). However, residual killing activity was observable at higher concentrations (2.0 to 5.0% milk). This activity was also stable to heating at 100°C for 20 min. The absence of killing with 1% heated milk and >50% killing with 2% heated milk may be related to the complex kinetics of killing observed previously [10]. These data are consistent with a two-component model for GCA (Fig. 3).

In preliminary studies with components of intestinal fluid, we observed that pancreatic lipases (but not proteases) killed G lamblia trophozoites in vitro. Therefore, it was of interest to test the two lipases of human milk.

TABLE II. Effects of Centrifugation and Dialysis Upon Giardiacidal Activity of Normal Human Milk

Treatment of milk	Parasite survival (% control)
Experiment A[a]	
Uncentrifuged	0
Pellet	0
Skim milk	0
Cream	0
Experiment B[b]	
Undialysed	13
Dialysed	0

[a]Survival was assayed by both direct microscopic and colony assays. No survival was observed by either assay at milk concentrations of 0.3% or greater.

[b]Survival was measured by colony assay. Control survival was 273 colonies/ml. The data shown are for 1% milk.

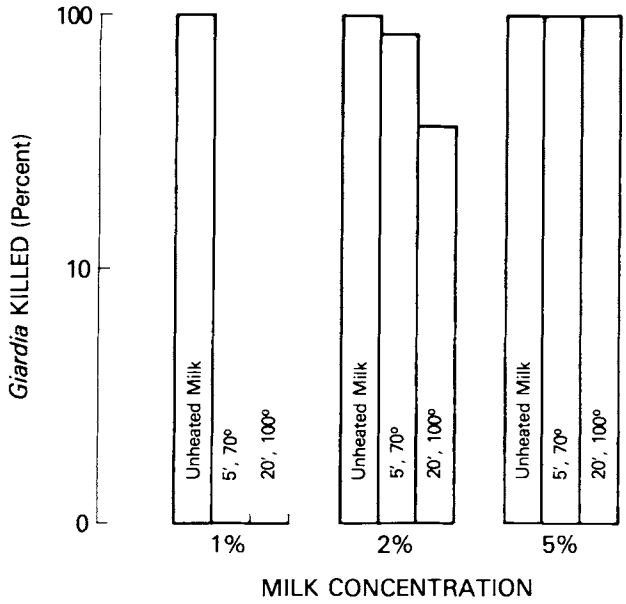

Fig. 2. Effect of heating on the Giardia-cidal activity of normal human milk. Milk samples were diluted tenfold into serum-free growth medium, heated at 70°C or 100°C for varying periods of time, and then chilled at 4°C. Parasites were incubated with varying concentrations of the milk samples for 3 h, and killing determined by colony assay. Killing by 0.3% heated or unheated milk was identical with that by 1% milk (not shown). Survival in controls was 1,723 colonies/ml.

In preliminary experiments, the bile salt-stimulated lipase (BSL) purified to apparent homogeneity from human milk killed >90% of G lamblia trophozoites in 3 h, but the lipoprotein lipase did not.

Since pure BSL killed G lamblia, we sought evidence that this enzyme had GCA in its "native" state in milk. BSL is a carboxylesterase that is inhibited by diisopropylfluorophosphate (DIFP), a specific irreversible inhibitor of serine esterases [20].

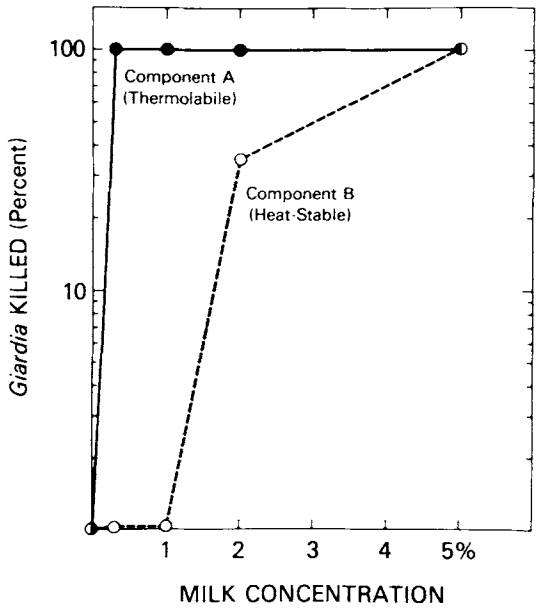

Fig. 3. Two-component model for killing G lamblia by normal human milk.

Modification by DIFP reduced the BSL esterase activity of an NHM sample by 90% and also greatly reduced its killing activity (Tables III, IV). Survival was increased threefold at 1% and >10 fold at 2% and 5% milk in DIFP-treated milk.

The decrease in killing activity observed after heating unfractionated milk (Fig. 2) was also accompanied by loss of BSL activity (Table IV). In addition, cow's milk and goat's milk lacked both BSL activity and GCA (Table IV).

These experiments support the idea that BSL has GCA, both in its pure form and in unfractionated human milk.

DISCUSSION

Our studies are the first demonstration of nonimmune antiparasitic activity in human milk. Giardia lamblia trophozoites were killed in a time- and concentration-dependent manner by NHM or milk depleted of secretory IgA antibodies [10].

In the presence of lethal concentrations of milk, the parasites rapidly lost the ability to attach, and flagellar beating and motility ceased. The trophozoites became rounded, granular, and ghostlike, and finally disappeared. The ultrastructural sequence and mechanism are not known. Two possibilities are that the plasma membrane was hydrolyzed so extensively that the parasites disintegrated or that interaction with the milk leads to autolysis.

It is likely that human milk contains more than one Giardia-cidal factor, as part of the activity was thermolabile, while the remainder was stable to 100°C for 20 min. Our data are consistent with a model in which NHM has both heat-labile (component A, Fig. 3) and less active or less abundant heat-stable (component B) Giardia-cidal factors. In other studies, the GCA was decreased but not eliminated by treating milk with DIFP, a specific serine esterase inhibitor. It is not known whether the heat-

TABLE III. Decreased Giardia-cidal Activity in Milk Reacted with DIFP*

Milk concentration (% vol/vol)	Survival (% control)[a]	
	DIFP-Modified	Unmodified
0	100	100
1	54.3	18.3
2	41.1	3.0
5	9.1	<0.02

*Five microliters of freshly diluted DIFP (Calbiochem) was added to a 1-ml sample of whole NHM to give a final concentration of 4×10^{-5} M DIFP. This is a 40-fold molar excess of DIFP to BSL based on an estimated concentration of 100 μg/ml (~ 10^{-6} M) BSL in the milk. The reaction was incubated on ice for 5 h, then unreacted DIFP was removed by overnight dialysis against PBS at 4°C. The unmodified milk was treated in the same way, except that it was never exposed to DIFP.

[a]Survival is expressed as % of controls lacking milk and was determined by the direct microscopic assay. 100% survival was 492 (±14) cells per field.

TABLE IV. Effect of Heat and DIFP Upon BSL Activity of Human Milk and Lack of Enzyme Activity in Cow's or Goat's Milk

	BSL Activity[a]
A. Human milk	
1. No Treatment[b]	3.5
10 min, 70°C	0.05
20 min, 100°C	0
2. No treatment[c]	20.0
DIFP	2.0
B. Cow's milk[b]	0
C. Goat's milk[b]	0

[a]Units/ml (1 unit = 1 μeq of fatty acid or p-nitrophenol released per min).

[b]Tributyrin assay.

[c]p-Nitrophenyl acetate assay.

resistant and DIFP-resistant activities are identical. Cow's and goat's milk were virtually devoid of GCA. This correlated with loss of activity of the major human milk lipase (BSL) upon heating and DIFP modification and absence of BSL from the milk of subprimate mammals [20,21]. In addition, BSL and killing activity cochromatographed on Sepharose 6B, and both were excluded from Sephadex G 100 [10]. These data support the idea that BSL may be a thermolabile Giardia-cidal component of milk.

BSL purified to apparent homogeneity also killed the parasites. The concentration of pure BSL required to kill the parasites increased at a time when both the enzyme purification method and the serum component of the parasite growth medium were changed. We are currently investigating the effects of these variables.

If BSL does prove to be a Giardia-cidal factor in vitro, it is likely that it would also be active in the small intestine of breast-fed babies. This enzyme survives exposure to conditions approximating passage through the infant stomach [21]. In the presence of bile salts, it assumes a protease-resistant conformation [21].

The identity of the heat-stable factor(s) is not known. It could be free fatty acids cleaved from milk triglycerides either spontaneously or by BSL, because free fatty acids are toxic. Other possibilities under consideration include human milk lactoferrin, lactoperoxidase, and xanthine oxidase, all of which are antibacterial.

Our results with human milk may have broader significance, as many antimicrobial factors in milk occur in other mucosal secretions. Indeed, we have proposed that some component(s) of this milieu may limit survival of this parasite in vivo [unpublished observations]. Giardia trophozoites were not adversely affected by intestinal or bacterial proteases, but were very sensitive to a pancreatic lipase preparation, which probably contained phospholipases (PL) A_2, C, and cholesterol ester hydrolase (which is related to BSL [22] as well as triacylglycerol lipase and colipase) [preliminary observations]. Thus, these parasites appear to be sensitive to a variety of lipases mucosal secretions.

It would be of great interest to know the effect of secretory or humoral antibodies on the Giardia-cidal activity of nonimmune human milk. Antibodies might either potentiate the killing by milk or could conceivably coat the parasites and protect them. Infant mice were protected from G muris infection by milk from previously infected mothers only [8]; however, rodent milk contains no BSL. Studies with secretory antibodies from human milk or intestinal fluid will require removal of lipase activities.

Although there is little data on the prevalence of intestinal parasitic protozoa in breast-milk- and formula-fed babies, "weanling diarrhea" is common, and in a study in Bangladesh [23], "exclusively breast-fed children seldom had giardiasis." Human milk would be more likely to be effective at preventing colonization of the small intestine by newly excysted G lamblia trophozoites than curing established infections in which many parasites would have penetrated the mucous layer and be protected.

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