ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1986, p. 254–257
0066-4804/86/080254-04$02.00/0

Vol. 30, No. 2

Killing of *Giardia lamblia* by Human Milk Is Mediated by Unsaturated Fatty Acids

LUCIA ROHRER,[1]* KASPAR H. WINTERHALTER,[2] JOHANNES ECKERT,[1] AND PETER KÖHLER[1]

Department of Parasitology, University of Zürich, 8057 Zurich,[1] and Department of Biochemistry, Swiss Federal Institute of Technology, Eidgenössiche Technische Hochschule, 8092 Zurich,[2] Switzerland

Received 5 December 1985/Accepted 7 May 1986

***Giardia lamblia* trophozoites were killed in vitro by 1% fresh human milk in the presence of bile. A similar effect was achieved in the absence of bile with milk which had been stored for at least 24 h at 6°C. This killing activity was found to be caused by unsaturated fatty acids. Depending on their chain length and the number of double bonds, the concentrations of unsaturated fatty acids required for a 50% killing effect varied between 15 and 36 μM. The saturated palmitic and stearic acids, as well as various substances related to fatty acids, showed only a slight killing effect. Bile enhanced and serum suppressed the action of fatty acids on the protozoan parasite. The possible site of interference of unsaturated fatty acids within *G. lamblia* and the reasons for the obvious inefficacy of dietary fatty acids in giardiasis are discussed.**

Giardia lamblia (synonym, *Giardia intestinalis*) is a binucleate flagellate and aerotolerant protozoan parasite which belongs to the order *Diplomonadia*. Infection of humans and mammals with *G. lamblia* is widespread, and the spectrum of manifestations ranges from asymptomatic passage of cysts to various forms of diarrhea with malabsorption (10). Infection of humans and animals alike with *G. lamblia* follows the ingestion of cysts from contaminated water or food (7, 8). After the ingestion of cysts, excystation occurs in an acidic environment, readily provided by the stomach. Subsequently, the trophozoites colonize the proximal small intestine, where they are exposed to digestive enzymes (i.e., pancreatic lipase), bile (which stimulates growth in vitro [18]), and nutrients.

Recently, Gillin et al. (12, 13) reported a parasiticidal effect of human milk on *G. lamblia* and suggested that the bile salt-stimulated lipase (BSSL) from milk was required for the killing effect. Results of this study provide evidence indicating that human milk-dependent killing of *G. lamblia* is caused by long-chain unsaturated fatty acids contained in the lipid fraction of milk.

MATERIALS AND METHODS

Microorganism. Trophozoites of *G. lamblia* WB (ATCC 30957) were cultured at 37°C in filter-sterilized TYI-S-33 medium (9), as modified by Keister (18). After 72 h of growth trophozoites were harvested by centrifugation for 10 min at 200 × *g* and washed twice in 8 mM potassium phosphate (pH 7.2) containing 0.85% NaCl.

Milk samples. Fresh bovine milk was obtained from the Institut für Zuchthygiene, University of Zürich. The fresh human milk was obtained from the Neonatology Unit, University Hospital Zürich, and originated from 14 different women between 23 and 35 years of age and from 1 to 40 days postpartum. The samples were used either immediately or after storage at 25, 6, −25, and −70°C.

Chemicals. Palmitic acid (hexadecanoic acid; $C_{16:0}$), stearic acid (octadecanoic acid; $C_{18:0}$), oleic acid (*cis*-9-octadecenoic acid; $C_{18:1}$), linoleic acid (*cis*-9-*cis*-12-octadecadienoic acid; $C_{18:2}$), linolenic acid (*cis*-9-*cis*-12-*cis*-15-octa-decatrienoic acid; $C_{18:3}$), elaidic acid (*trans*-9-octadecenoic acid), oleyl alcohol (*cis*-9-octadecen-1-ol), methyl oleat (methyl-*cis*-9-octadecenoate), ethyllinoleate (ethyl-*cis*-9-*cis*-12-*cis*-15-octadecatrienoate), and (+)-α-tocopherol were purchased from Fluka, Buchs, Switzerland, and were of the highest available purity. Bile (bovine bile B-8381), a mixture of free and conjugated bile acids, was obtained from Sigma Chemical Co., St. Louis, Mo.

Determination of fatty acids in milk samples. Free fatty acids in fresh and stored human milk were extracted by a modified method of Folch (19) with the following solvent system: $CHCl_3$-CH_3OH (2:1 [vol/vol]). The fatty acids were determinated quantitatively as the methyl esters by gas chromatography.

Susceptibility test. The protozoans were suspended in the culture medium, but without bile and serum, and adjusted to a cell density of 6×10^5 to 8×10^5 per ml. All experiments were initiated by the addition of either milk or fatty acids to test tubes containing 1 ml of parasite suspension and other test substances appropriately diluted with the culture medium described above. The final assay volume was 1.5 ml, containing 4×10^5 to 6×10^5 trophozoites per ml. The fatty acids and related compounds were tested at the following concentrations (in nanomoles per milliliter [micromolar]): 2, 5, 10, 20, 30, 40, 50, 100, 150, 200, 250, 300, 400, 500, 1,000. Control incubations lacking milk, fatty acids, and other test components were included in each experiment. The test tubes were mixed carefully by inversion and then incubated for 3 h at 37°C. Unless otherwise indicated, the experiments were terminated by chilling the tubes on ice for 15 min. The numbers of living *Giardia* cells in untreated controls (at least five per experiment) and in treated samples were determined by microscopic counting of the motile organisms in a Neubauer hematocytometer. Dead trophozoites, if visible, could be easily recognized as immobile cells which were accompanied by swelling and discharge of cytoplasmic components. Survival was expressed as the average percentage of living organisms in treated cultures in relation to untreated ones. These controls contained 4×10^5 to 6×10^5 cells and were taken as a 100% reference. Each experiment was repeated three to six times.

* Corresponding author.

TABLE 1. Effect of human milk on *G. lamblia* trophozoites in vitro

Expt	Preincubation of milk	% final concn of test medium supplemented with:			% trophozoite survival[a]
		Milk	Bile	Serum	
A	Control, none	0	0	0	100 (25)
		1	0	0	96 (25)
		5	0	0	88 (3)
		1	0	10	98 (25)
		1	0.05	0	0 (25)
		1	0.05	10	98 (25)
B	Incubation, 3 min, 80°C	1	0	0	95 (5)
		1	0	10	98 (5)
		1	0.05	0	95 (5)
		1	0.05	10	97 (5)
C	Incubation, 3 h, 37°C, mixed with supplements	1	0	0	99 (5)
		1	0	10	100 (5)
		1	0.05	0	0 (5)
		1	0.05	10	8 (5)
D	Incubation, 3 min, 80°C, and then 3 h, 37°C, mixed with supplements	1	0	0	99 (5)
		1	0	10	100 (5)
		1	0.05	0	96 (5)
		1	0.05	10	98 (5)

[a] Number of experiments is given in parentheses; standard deviation was always less than 20%.

TABLE 2. Influence of storage on the concentration of fatty acids in human milk and the consequences for *G. lamblia*

Storage time (h) at 6°C	Total fatty acids in milk (mg/liter)	Distribution of unsaturated fatty acids (μM)			Concn of unsaturated fatty acids in test (μM)	Effect on *G. lamblia*
		$C_{18:1}$	$C_{18:2}$	$C_{18:3}$		
0	250	273	178	11	5.3	None
12	290	353	215	55	7.0	None
24	1,380	1,140	553	184	24.2	Lethal
48	3,850	6,220	3,340	368	107.8	Lethal
72	5,530	8,777	4,920	549	154.0	Lethal

RESULTS

A very slight *G. lamblia*-cidal effect was observed when the trophozoites were incubated in the presence of 5% fresh human milk, whereas 1% was essentially ineffective (Table 1, experiment A). In the presence of bile, however, supplementation of the test samples with 1% fresh human milk resulted in complete killing of the *Giardia* population (experiment A). This effect was abolished by the addition of serum. No lethal effect was found when the organisms were incubated in the presence of bile and milk which previously had been inactivated for 3 min at 80°C (Table 1, experiments B and D). However, under similar conditions, the parasites were destroyed by milk which had been preincubated with bile for 3 h at 37°C (experiment C). Under the conditions of experiment C the protective effect of serum was only marginal. Interestingly, no *G. lamblia*-cidal effect was observed on replacement of human milk by bovine milk, not even after preincubation for 6 days at 25°C (data not shown).

Our assumption that the observed killing effect of human milk was produced by free fatty acids was corroborated by chemical analyses of milk samples. Free fatty acid contents increased considerably during storage (Table 2). The main unsaturated component in the milk sample of a 28-year-old woman, 12 days postparturition, was oleic acid, followed by linoleic and linolenic acids. In agreement with previously published results (19), further analyses have shown that lipid content and composition in the milk is influenced by nutritional status. Additional experiments demonstrated that the killing activity of fresh and inactivated human milk could be initiated by supplementing unsaturated fatty acids in the same quantity as found in human milk (Table 2).

Analogous results were obtained with fresh and inactivated bovine milk. The effect of a variety of long-chain fatty acids on the vitality of *G. lamblia* is summarized in Fig. 1. Concentrations of unsaturated fatty acids necessary for a 50% killing effect varied between 15 and 36 μM, the more unsaturated fatty acids being more active. The saturated stearic acid showed only a slight activity even at excessively

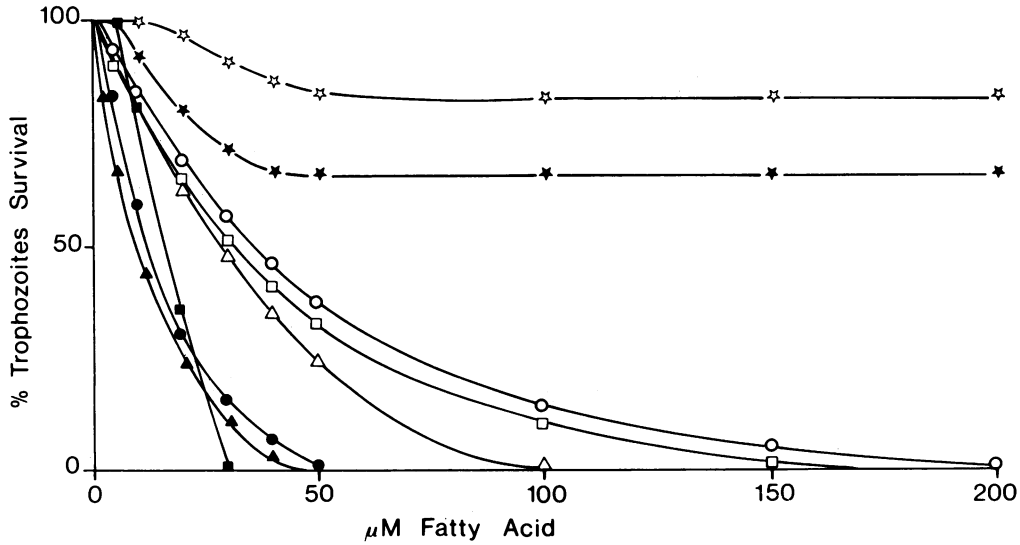

FIG. 1. *G. lamblia*-cidal effect of fatty acids in the presence of bile (closed symbols) and in the absence of bile (open symbols). For clarity not all data points are shown. The standard deviation of the means was a maximum of 20%. Symbols: ☆ and ★, stearic ($C_{18:0}$); △ and ▲, linolenic ($C_{18:3}$); □ and ■, linoleic ($C_{18:2}$); ○ and ●, oleic acid ($C_{18:1}$).

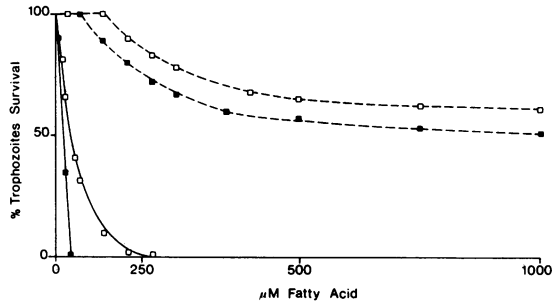

FIG. 2. *G. lamblia*-cidal activity of linoleic acid ($C_{18:2}$) in the absence (——) and presence (----) of serum, and in the absence (□) and presence (■) of bile.

high concentrations, and a similar finding was obtained with palmitic acid. The effect of unsaturated fatty acids on *G. lamblia* was enhanced by bile (Fig. 1), while serum suppressed the action of the acids on the organisms (Fig. 2). Only a small *G. lamblia*-cidal effect was found by incubation with various compounds related to fatty acids (Fig. 3). Interestingly, the replacement of the *cis* configurational isomer oleic acid by the *trans* form (elaidic acid) resulted in a reduced killing effect (Fig. 3). Further studies demonstrated that the killing activity was not markedly changed at reduced oxygen pressures or when tocopherol, a radical scavenger, was added to the test medium (data not shown).

DISCUSSION

Results of this study support the conclusion that the *G. lamblia*-cidal effect produced by human milk is due to the action of long-chain unsaturated fatty acids. In human milk these acids are formed by activation of BSSL or during milk storage. In contrast, no killing effect was found with bovine milk, in which free fatty acid formation is absent (3, 4, 21).

Killing activity of fresh human milk in the absence of bile salts, as first reported by Gillin et al. (12, 13), was not observed in this study. A possible explanation for this discrepancy could be that Gillin et al. did not use entirely fresh milk, so that lipolysis may have occurred (Table 2) (1–3, 5, 6, 14, 20). In addition, Gillin et al. (12, 13) have found a similar *G. lamblia*-cidal effect by incubating *G. lamblia* in the presence of BSSL purified from human milk or, alternatively, by treating the organisms with a pancreatic lipase preparation which was found to contain a BSSL-related activity. Recently Gillin et al. (11) have amended their previously obtained results and have reported that the observed killing activity of human milk against *G. lamblia* is, in fact, dependent on the presence of sodium cholate (bile salt), which is in agreement with our results. Furthermore, we observed a lethal effect on the protozoan parasites by treatment with pure, long-chain, unsaturated fatty acids. The magnitude of the effect was dependent on the nature of the fatty acid tested. Free fatty acids were found in the lipid fraction of human milk (19) but not in bovine milk; indeed, the latter showed killing activity only on the addition of free fatty acids.

These results demonstrate that the *G. lamblia*-cidal activity was not dependent on BSSL or antibody.

The effect of unsaturated fatty acids was enhanced by bile, possibly through its detergentlike action. Interestingly, the deleterious effect paralleled the degree of unsaturation. The protective effect of serum is most easily rationalized by its content of free fatty acid-binding proteins such as albumin. Indeed, incubation of *G. lamblia* with [^{3}H]oleic acid resulted in the binding of more than 60% of the radioactivity to the organisms separated from the medium by centrifugation. If serum (10%) was present, less than 10% bound to the pellet fraction (unpublished data). The killing activity of unsaturated fatty acids was so dependent on the geometric arrangement of the double bond that the *trans*-configurational isomer was virtually inactive. This is in keeping with the suggestion that fatty acids interact with the trophozoite membrane in a stereochemically specific way. The lower *G. lamblia*-cidal activity observed with compounds related to unsaturated fatty acids indicated that the free carboxylic group was important for this effect.

Despite the sensitivity of *G. lamblia* toward long-chain fatty acids in vitro, the organisms readily survive in an in vivo environment containing an abundant supply of these substances. This obvious discrepancy is explained by the

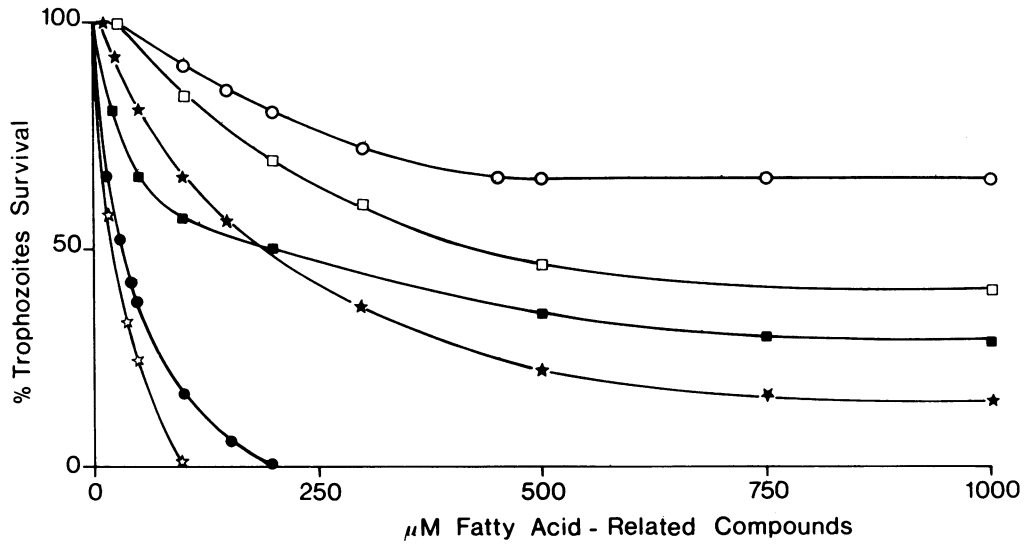

FIG. 3. Effect of fatty acids and related compounds on *G. lamblia*. For clarity not all data points are shown. The standard deviation of the means was a maximum of 20%. Symbols: ●, oleic acid (*cis*-$C_{18:1}$); ○, elaidic acid (*trans*-$C_{18:1}$); □, methyl oleat; ■, oleyl alcohol; ☆, linolenic acid ($C_{18:3}$); ★, ethyllinoleate.

fact that products of lipolysis in the small intestine are dispersed in liposomes or bile micelles for absorption by enterocytes and therefore are not available in free form in sufficient concentrations. Alternatively, the bacterial flora of an intestine occupied by *G. lamblia* may be unusual in that it can give rise to the deconjugation of bile (22). As a consequence, fatty acids may loose their destructive ability.

To our knowledge this is the first report on the killing activity of fatty acids against a protozoan parasite. Although the biological activity of these compounds has been previously demonstrated for viruses, bacteria, and yeasts, its molecular basis is as yet unknown (15–17, 23). It has been suggested, however, that long-chain fatty acids and their derivatives can affect unicellular organisms by interacting with their lipid membranes, possibly resulting in perturbations of the lipid phase, thereby causing changes in membrane permeability. Because of their direct exposure to the host, the cell surface structures of parasitic organisms appear to be very particular sites for chemotherapeutic attack. Thus, the observed specific effect of physiologically occurring long-chain unsaturated fatty acids against *G. lamblia* may open up a new approach for drug development against the causative agent of the widespread disease caused by this protozoan parasite.

ACKNOWLEDGMENTS

This work was supported by Swiss National Science Foundation grant 3.89.0.83.

Human milk samples were the generous gift of J. Micallef, Neonatology Unit, University Hospital Zürich. We thank A. Prabucki, Animal Production, Swiss Federal Institute of Technology, Eidgenössiche Technische Hochschule, Zurich, for determination of the fatty acids in human milk. We are grateful to T. Nash, National Institutes of Health, Bethesda, Md., and D. Warhurst, London School of Hygiene and Tropical Medicine, London, for helpful advice on culture techniques. We also thank Karin Schwarz for excellent technical assistance.

LITERATURE CITED

1. **Berkow, S. E., L. M. Freed, M. Hamosh, J. Bitman, D. L. Woo, B. Happ, and P. Hamosh.** 1984. Lipases and lipids in human milk: effect of freeze-thawing and storage. Pediatr. Res. **18**:1257–1262.
2. **Bitman, J., D. L. Wood, N. R. Metha, P. Hamosh, and M. Hamosh.** 1983. Lipolysis of triglycerides of human milk during storage at low temperatures: a note of caution. J. Pediatr. Gastroenterol. Nutr. **2**:521–524.
3. **Bläckberg, L., D. Lombardo, O. Hernell, O. Guy, and T. Olivecrona.** 1981. Bile salt-stimulated lipase in human milk and carboxylester hydrolase in pancreatic juice. FEBS Lett. **136**:284–288.
4. **Castberg, H. B., and O. Hernell.** 1975. Role of serum stimulated lipase in lipolysis in human milk. Milchwissenschaft **30**:721–724.
5. **Clark, R. M., K. E. Hundrieser, P. B. Brown, M. Lavine, and R. G. Jensen.** 1984. The effect of temperature and length of storage on bile salt-stimulated lipase and esterase in human milk. Nutr. Res. **4**:957–960.
6. **Clark, R. M., K. H. Hundrieser, S. Ross, and P. B. Brown.** 1984. Effect of temperature and length of storage on serum-stimulated and serum-independent lipolytic activities in human milk. J. Pediatr. Gastroenterol. Nutr. **3**:567–570.
7. **Danciger, M., and M. Lopez.** 1975. Number of *Giardia* in the feces of infected children. Am. J. Trop. Med. Hyg. **24**:237–242.
8. **Davies, R. B., and C. P. Hibler.** 1979. Animal reservoirs and cross-species transmission of *Giardia*, p. 104–126. *In* W. Jakubowski and J. C. Hoff (ed.), Waterborne transmission of giardiasis. U.S. Environmental Protection Agency, Cincinnati, Ohio.
9. **Diamond, L. S., D. R. Harlow, and C. C. Cunnick.** 1978. A new medium for axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Trans. R. Soc. Trop. Med. Hyg. **72**:431–432.
10. **Feely, D. E., S. L. Erlandsen, and D. G. Chase.** 1984. Structure of the trophozoite and cyst, p. 5–50. *In* S. L. Erlandsen and E. A. Meyer (ed.), *Giardia* and giardiases. Plenum Publishing Corp., New York.
11. **Gillin, F. D., D. S. Reiner, and M. J. Gault.** 1985. Cholate-dependent killing of *Giardia lamblia* by human milk. Infect. Immun. **47**:619–622.
12. **Gillin, F. D., D. S. Reiner, and C. S. Wang.** 1983. Killing of *Giardia lamblia* by normal human milk. J. Cell. Biochem. **23**:47–56.
13. **Gillin, F. D., D. S. Reiner, and C. S. Wang.** 1983. Human milk kills parasitic intestinal protozoa. Science **221**:1290–1292.
14. **Hamosh, A., S. Berkow, J. Bitman, L. Freed, B. Happ, J. B. Jones, N. R. Metha, D. L. Wood, and P. Hamosh.** 1984. Handling and storage of human milk species for research. J. Pediatr. Gastroenterol. Nutr. **3**:284–289.
15. **Kabara, J. J.** 1978. Fatty acids and derivates as antimicrobial agents, p. 1–13. *In* J. Kabara (ed.), The pharmacological effect of lipid. The American Oil Chemists' Society, Champaign, Ill.
16. **Kabara, J. J.** 1980. Lipids as host resistance factors of human milk. Nutr. Rev. **38**:65–73.
17. **Kabara, J. J., R. Vrable, and M. S. F. Lie Ken Jie.** 1977. Antimicrobial lipids: natural and synthetic fatty acids and monoglycerides. Lipids **12**:753–759.
18. **Keister, D. B.** 1983. Axenic culture of *Giardia lamblia* in TYI-S-33 medium supplemented with bile. Trans. R. Soc. Trop. Med. Hyg. **77**:487–488.
19. **Lammi-Keefe, C. J., and R. G. Jensen.** 1984. Lipids in human milk. 1. A review. 2. Composition and fat-soluble vitamins. J. Pediatr. Gastroenterol. Nutr. **3**:172–198.
20. **Metha, N. R., J. B. Jones, and M. Hamosh.** 1982. Lipases in preterm human milk: ontogeny and physiologic significance. J. Pediatr. Gastroenterol. Nutr. **1**:317–326.
21. **Olivecrona, T., and G. Bengtsson.** 1983. Lipases in milk, p. 207–250. *In* B. Borgström and H. L. Brockmann (ed.), Lipases. Elsevier, New York.
22. **Smith, P. D., C. R. Horsburg, and W. R. Brown.** 1981. In vitro studies on bile acid deconjugation and lipolysis inhibition by *Giardia lamblia*. Digest. Dis. Sci. **26**:700–704.
23. **Thomson, A. B. R., and J. M. Dietschy.** 1981. Intestinal lipid absorption: major extracellular and intracellular events, p. 1147–1220. *In* Physiology of the gastro-intestinal tract. Raven Press, New York.