Addition of lipases to infant formulas produces antiviral and antibacterial activity

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Six infant formulas in which triglycerides varied in the proportions of their constituent medium-chain saturated and long-chain unsaturated fatty acids were incubated with either pancreatic lipase, pancreatic lipase plus colipase, bile salt-stimulated lipase, or lipoprotein lipase and then measured for antiviral and antibacterial activity. Herpes simplex virus-1 (HSV-1) was inactivated following all incubations, with the exception of lipoprotein lipase, with one of three infant formulas with a coconut/soy blend (formula 5). After incubation of each formula with pancreatic lipase plus colipase, dilutions of these mixtures by as much as 100–250 fold still inactivated HSV-1. Lipase-treated formulas inactivated the gram-positive bacterium Staphylococcus epidermidis *but were ineffective against gram-negative* Escherichia coli *and* Salmonella enteritidis. *The present study shows that the lipid fraction of infant formulas is not only a source of nutrients but also a source of antiviral and antibacterial activity following incubation with lipases.*

Keywords: herpes simplex virus-1; *Staphylococcus epidermidis; E. coli; Salmonella enteritidis;* infant formula; lipases

Introduction

The action of endogenous lipases on the lipid fraction of human milk during storage at 4° C releases antimicrobial fatty acids and monoglycerides from milk triglycerides.[1] The production of antimicrobial lipids also occurs in the gastrointestinal tract following the digestion of milk triglycerides by lingual and gastric lipases,[2–5] as demonstrated by the strong antimicrobial activity of lipid extracts from the stomach contents of human milk-fed infants.[6]

Infant formulas are designed to provide nutrients to infants, but do not contain components that provide protection against infection. However, triglycerides in infant formula consist of long-chain unsaturated and medium-chain saturated fatty acids that have been shown to be antimicrobial when present in the form of monoglycerides or free fatty acids.[7,8] Recently, we have shown that the lipid fraction extracted from the stomach contents of formula-fed infants is, in fact, antimicrobial.[6]

The present study was undertaken to determine whether formula lipids contribute to the antimicrobial activity found in the lipid extracts of stomach contents from formula-fed infants. Infant formulas were incubated with a number of different lipases in vitro and measured for antiviral and antibacterial activity.

Materials and methods

Incubation of formulas with lipases

Experimental whey-predominant formulas differing in fat source and fatty acid composition, yet similar in composition to commercial infant formulas, with nutritionally complete levels of protein, fat lactose, vitamins, and minerals *(Table 1)* were incubated with various lipases under conditions previously described[9] for lipase assays. The fatty acid profile

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Table 1 Triglyceride and monoglyceride composition of experimental milk-based infant formulas[a]

Lipid composition	Test Formula[b]					
	1	2	3	4	5	6
Fat blend[c]	0:45:40:15	20:20:40:20	0:60:40:0	55:45:0:0	55:45:0:0	55:45:0:0
g triglycerides/L[d]	37.58	37.58	37.58	36.22	36.35	37.58
g monoglycerides/L[e]	0.75	0.75	0.75	0.58	0.45	0.75
Triglyceride fatty acid distribution (% wt/wt)						
C6	0	0.1	0	0.28	0.28	0.28
C8	0	1.6	0	4.4	4.4	4.4
C10	0	1.2	0	3.3	3.3	3.3
C12	0.1	9.3	0.2	25.4	25.4	25.4
C14	0.5	4.1	0.5	9.9	9.9	9.9
C16	22.9	22.4	23.7	9.5	9.5	9.5
C18	3.9	3.5	4.4	3.6	3.6	3.6
C18:1	37.5	36.6	29.9	14.9	14.9	14.9
C18:2	30.4	18.3	36.3	25.2	25.2	25.2
C18:3	3.5	1.6	4.7	3.5	3.5	3.5
C20	0.2	0.2	0.2	0	0	0

[a]The fatty acid composition of the formulas was provided by the manufacturer based on analysis of the separate oils used to make the formula.
[b]Whey predominate milk-based infant formulas containing nutritionally complete levels of protein, fat, lactose, vitamins, and minerals.
[c]Given as the weight ratio of coconut:soy:palm:high oleic safflower oils.
[d]Concentration in liquid infant formulas with a caloric density of 670 kcal/L.
[e]Present in the formula for fat emulsification.

of the triglycerides in the formulas was calculated from the average analytical values determined separately for each of the individual fat ingredients.[10] It does not include the monoglyceride concentrations. The assay mixture for bile salt-stimulated lipase (BSSL) was 60 mmol/L Tris-HCl (pH 8.6), 2.8% bovine serum albumin (BSA), 12 mmol/L sodium taurocholate, 5 μg of BSSL (specific activity 90 μmol min^{-1} mg^{-1}), and 500 μL infant formula in a final volume of 1 mL. Incubation was for 1 hour at 37° C, after which samples were put on ice, aliquoted, and frozen at −86° C. Pancreatic lipase was also incubated for 1 hour at 37° C in a mixture containing 200 mmol/L Tris buffer (pH 8.0), 30 μmol/L BSA, 6 mmol/L $CaCl_2$ 8 mmol/L sodium taurocholate, 150 mmol/L NaCl, 10 μg of porcine pancreatic lipase, and 500 μL infant formula in a total volume of 1 mL. Colipase was added at a concentration of 10 μg/mL when it was used with pancreatic lipase. The lipoprotein lipase (LPL) mixture, in a final volume of 1 mL incubated for 1 hour at 37° C, contained 200 mmol/L Tris-HCl (pH 8.6), 5% BSA, 0.25 U of heparin, 3 μg of LPL (specific activity 622 μmol min^{-1} mg^{-1}) and 500 μL infant formula. The lipase-treated infant formulas were then assayed for antimicrobial activity as described below. Assay mixtures with lipases, but without added infant formula, were used as a control.

Assay of antiviral activity

Viral infectivity was measured by inoculation of 10-fold dilutions of Herpes simplex virus type 1 (HSV-1), strain MacIntyre, and vesicular stomatitis virus, strain Indiana, into Vero cell cultures (African green monkey kidney cell line; Flow Laboratories, Inc., McLean, VA, USA) in 96-well microtiter tissue culture plates (Becton Dickinson Labware, Oxnard, CA, USA). Vero cells were grown in Eagle basal medium (BME) (GIBCO Laboratories, Grand Island, NY, USA) with 10% inactivated fetal bovine serum (GIBCO). The maintenance medium (MM) for Vero cells was BME with 2% fetal bovine serum and 0.1% Gentamicin (Whittaker Bioproducts, USA). A virus dilution (0.1 mL) in MM was inoculated into each of four wells per dilution. The plates were kept for 4 days and examined daily for cytopathic effect. Virus titers were calculated by the method of Reed and Muench.[11]

Antiviral activity of lipase-treated infant formulas were assayed by mixing about 10^5 tissue culture infective doses ($TCID_{50}$) of virus with a five-fold dilution of lipase-treated infant formula, and incubated at 30° C for 30 min. Virus mixed with MM or formula alone was used as a control. After incubation, the infectivity of each mixture was titrated by the serial dilution endpoint method. The 10^{-2}–10^{-5} dilutions were inoculated into monolayers of Vero cells and the virus titers were determined as described above. The difference between the titer (log_{10}) of virus in the control MM and the titer (log_{10}) of virus in lipase-treated infant formulas, i.e., the log reduction of virus titer, was used as a measure of antiviral activity.

Assay of antibacterial activity

The bacteria to be tested were adjusted to concentrations of 10^4/mL to 10^5/mL and mixed with an equal volume of lipase-treated infant formula. After incubating for 1 hour at 37° C, the bacteria plus formula mixtures were diluted in 10-fold increments, plated on nutrient agar or sheep blood agar (Scott Laboratories, Fiskeville, RI, USA), and incubated overnight at 37° C at which time the colonies were counted. Bacterial inactivation was determined by subtracting the number of colonies on plates of sample-treated bacteria from the number on control plates.

Purified BSSL from human milk[12] and LPL from bovine milk[13] were gifts from Dr. Olle Hernell and Dr. Lars Blackberg (Department of Physiology Chemistry, University of Umea, Sweden). Pancreatic lipase and colipase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Herpes simplex virus type 1, strain MacIntyre, was obtained from the American Type Culture Collection (Rockville, MD,

USA). All bacterial strains *(Salmonella enteritidis, Staphylococcus epidermidis* and *Escherichia coli)* were obtained from the Microbiology Laboratory of the Consolidated Clinical Laboratories (N.Y.S. Institute for Basic Research, Staten Island, NY, USA).

Results

Six infant formulas were treated with lipases and examined for antiviral and antibacterial activity. Incubation of the formulas, diluted 1:5 with pancreatic lipase, resulted in a greater than 4.5 $\log_{10}$ reduction in antiviral activity *(Table 2)*. The soy- and palm oil-predominant formulas 1–3 had antiviral activity lowered by 2.7 or 2.0 $\log_{10}$ when diluted 25 fold, while the coconut and soy oil formulas 4–6 had no change in inactivation when diluted 25 fold compared to five-fold dilutions. A 1:50 dilution of formulas 1 and 2 decreased the antiviral activity to zero, while formulas 3–6 retained some antiviral activity ranging from 0.5–3.3 $\log_{10}$ reduction in HSV-1 titers.

When colipase was added with the pancreatic lipase prior to incubation with the formulas *(Table 2)*, antiviral potency was even greater than with pancreatic lipase by itself at dilutions greater than five fold. A 100-fold dilution of formulas 1, 2, and 4 treated with pancreatic lipase and colipase did not decrease antiviral activity as was observed when the formulas diluted 50 fold were treated with pancreatic lipase alone. Formulas 3, 5, and 6 only lost approximately one-half of a log of antiviral activity when diluted 100 fold. Even when diluted 1:250, each formula retained at least 1.2 $\log_{10}$ reduction of antiviral activity.

Infant formulas incubated with BSSL *(Table 2)* also produced antiviral activity. Formula 1 treated with BSSL had maximum antiviral activity at the 1:5 dilution and lost all antiviral activity at the 1:50 dilution, the same result as with formula 1 incubated with pancreatic lipase. Formulas 2 and 3 diluted 1:5 decreased $\log_{10}$ reductions of HSV-1 titers to 0.5 and 1.5, respectively. Formulas 4–6 diluted 1:50 had antiviral activity comparable to formula treated with pancreatic lipase at the same dilution.

Lipoprotein lipase-treated formulas had less antiviral activity than the other lipase-treated formulas *(Table 2)*. Formula 5 undiluted did not have antiviral activity and formula 4 undiluted only showed a 1.5 $\log_{10}$ decrease in HSV-1 titer. When diluted 1:5, all the formulas had little or no detectable antiviral activity.

All six infant formulas, following incubation with pancreatic lipase or pancreatic lipase plus colipase, were mixed undiluted in equal volume with either gram-positive or gram-negative bacteria *(Table 3)*. Maximum antibacterial activity ($\geq$4.0 $\log_{10}$ reduction) was found against gram-positive *S. epidermidis*, but no antibacterial activity was observed against gram-negative *E. coli* and *S. enteritidis*.

Discussion

The results of this study show that infant formulas containing varying proportions of triglycerides of medium-chain saturated and long-chain unsaturated fatty acids *(Table 1)* develop antiviral and antibacterial activity following incubation with lipases *(Tables 2 and 3)*. This is in agreement with a previous study[7] that found that both purified medium-chain saturated, e.g., capric (10:0), and long-chain unsaturated fatty acids, e.g., oleic (18:1), have antiviral activity. Formulas 1 and 3 *(Table 1)* have triglycerides with antiviral levels of oleic (18:1), linoleic (18:2), and linolenic (18:3) acids. Formulas 2, 4, 5, and 6 contain the above three antiviral fatty acids as well as lauric acid (12:0) and capric acid (10:0), which are also antiviral.[7] If complete hydrolysis occurs, each formula will produce a total concentration of antimicrobial fatty acids ranging from 90–100 mmol/L. Measurements of triglycerides and free fatty acids in the gastric contents taken from infants fed medium-chain triglyceride or long-chain triglyceride-containing formulas,[14] similar to the ones used in this study, showed that both types of formulas produced antimicrobial levels of free fatty acids following gastric lipolysis when the total concentration of medium-chain saturated and long-chain unsaturated free fatty acids was considered. While there are antiviral monoglycer-

Table 2 Inactivation of HSV-1 by infant formulas treated with lipases[a,b]

	Pancreatic lipase			Pancreatic lipase + colipase			Bile salt-stimulated lipase			Lipoprotein lipase	
	Dilutions of lipase-treated formula										
Formula	1:5	1:25	1:50	1:5	1:100	1:250	None	1:5	1:50	None	1:5
1	≥4.5	2.0	0	≥4.5	≥4.5	1.8	—[c]	≥4.5	0	≥4.5	1.5
2	≥4.5	2.0	0	≥4.5	≥4.5	1.8	≥4.5	0.5	—	≥4.5	0.5
3	≥4.5	2.7	0.5	≥4.5	4.0	1.2	≥4.5	1.5	—	≥4.5	1.7
4	≥4.5	≥4.5	3.3	≥4.5	≥4.5	2.0	—	≥4.5	≥3.5	1.5	0.5
5	≥4.5	≥4.5	2.5	≥4.5	4.0	1.8	—	≥4.5	3.5	0	0
6	≥4.5	≥4.5	3.3	≥4.5	4.0	2.2	—	≥4.5	3.7	≥4.5	0.7

[a]Inactivation expressed as the $\log_{10}$ decrease from the initial HSV-1 titer ($TCID_{50}$) after incubation with different dilutions of infant formulas treated with lipases.
[b]Antiviral activity was not detected in any of the formulas prior to incubation with lipase(s).
[c]Not done.

Table 3 Bacterial inactivation by infant formulas treated with lipases[a,b]

	Pancreatic lipase[c]			Pancreatic lipase + colipase[d]		
Formula	*E.coli*	*S.enteritidis*	*S.epidermidis*	*E.coli*	*S.enteritidis*	*S.epidermidis*
1	0	0	2.74	0	0	3.08
2	0	0	2.71	0	0	2.90
3	0	0	1.91	0	0	2.64
4	0	0	1.87	0	0	2.74
5	0	0	1.91	0	0	2.70
6	0	0	1.53	0	0	2.60

[a]Inactivation expressed as the $\log_{10}$ decrease from the initial bacteria concentration after incubation with undiluted infant formula treated with lipase(s).
[b]Antibacterial activity was not detected in any of the formulas prior to incubation with lipase(s).
[c]The initial bacterial concentrations for *E. coli, S. enteritidis,* and *S. epidermidis* were 2.7×10^4/mL, 1.2×10^5/mL and 6.0×10^4/mL, respectively.
[d]The initial bacterial concentrations for *E. coli, S. enteritidis,* and *S. epidermidis* were 1.3×10^5/mL, 1.4×10^5/mL and 3.1×10^4/mL, respectively.

ides in formulas 4 and 5, their total concentration is approximately 100 μmol/L and therefore insufficient to produce antimicrobial activity.[7] However, when antimicrobial fatty acids and monoglycerides are released from triglycerides by hydrolysis, monoglycerides already present can add to the total antimicrobial activity.[5] This study supports an earlier suggestion that moderate changes in the proportions of human-milk medium-chain saturated and long-chain unsaturated fatty acids, as the result of modification of the maternal diet,[15,16] would not decrease antimicrobial activity in milk lipid fractions[17] because it is the total concentration of antimicrobial lipids in solution that is critical and not one particular fatty acid.[5]

Studies that examined the stomach contents of formula-fed infants suggest that following lipolysis in the infant's gastrointestinal tract all formulas develop comparable antiviral and antibacterial activity.[6] However, the persistence of antiviral activity following formula dilution varied with the formula and lipase combination in the present study. This may reflect different degrees of lipolysis occurring in the formulas as well as different fatty acid compositions. The higher antiviral potency with greater formula dilution when pancreatic lipase was combined with colipase is likely due to more complete hydrolysis of the formula triglycerides than by pancreatic lipase alone.

Infant formulas treated with lipase in this study killed only gram-positive *S. epidermidis,* but neither of the two gram-negative bacteria. However, the stomach contents of formula-fed infants inactivated both gram-positive and gram-negative bacteria.[2] Because gram-negative bacteria are more resistant to inactivation by medium- and long-chain fatty acids than gram-positive bacteria,[18] the difference between the in vivo and in vitro results may reflect incomplete lipolysis of formula triglycerides in the incubation mixtures. It is also possible that the gram-negative bacterial killing, found with stomach contents samples, requires mucosal surface lipids in the infant's gastrointestinal tract that work independently or in conjunction with formula lipids. For example, it has been shown that lipids in human proximal intestinal fluid[19] inactivate *Giardia lamblia* trophozoites and that fatty acids released at other secretory surfaces such as lung[20] and skin[21] have antimicrobial activity.

Infant formulas are reported to support the growth of bacterial pathogens in vitro as opposed to human milk, which inhibits their growth.[22] This difference may be due to the presence of lipases in milk that liberate antimicrobial fatty acids and monoglycerides from triglycerides during storage as well as the presence of antibodies and nonspecific protective factors in milk, e.g., lysozyme and lactoferrin, which are lacking in formula. The results presented here, however, suggest that infant formula develops antimicrobial activity following the hydrolysis of triglycerides during digestion and therefore may inactivate pathogenic organisms in the gastrointestinal tract.

In conclusion, infant formulas with lipids, mainly in the form of triglycerides, can develop antimicrobial activity against HSV-1 and *S. epidermidis* (but not gram-negative bacteria) following hydrolysis by various lipases in vitro. Further studies are being conducted to determine whether the addition of monoglycerides and fatty acids to formula prior to digestion will yield antimicrobial activity.

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References

1 Isaacs, C.E., Thormar, H., and Pessolano, T. (1986). Membrane-disruptive effect of human milk: Inactivation of enveloped viruses. *J. Infect. Dis.* **154,** 966–971
2 Fink, C.S., Hamosh, P., and Hamosh, M. (1984). Fat digestion in the stomach: Stability of lingual lipase in the gastric environment. *Ped. Res.* **18,** 248–254
3 Liao, T.H., Hamosh, P., and Hamosh, M. (1984). Fat digestion by lingual lipase: Mechanism of lipolysis in the stomach and upper small intestine. *Ped. Res.* **18,** 402–409

4 Isaacs, C.E., and Thormar, H. (1990). Human milk lipids inactivate enveloped viruses. In *Breastfeeding, Nutrition, Infection and Infant Growth in Developed and Emerging Countries,* (S.A. Atkinson, L.A. Hanson, R. K. Chandra and R.K. Chandra, eds.), p. 161–174, Arts Biomedical Publishers and Distributors, St. John's, Newfoundland, Canada

5 Hamosh, M. (1987). Lipid metabolism in premature infants. In *Energy Metabolism, Nutrition and Growth in Premature Infants, Biol. Neonate* **52** (Suppl. 1), 50–64

6 Isaacs, C.E., Kashyap, S., Heird, W.C., and Thormar, H. (1990). Antiviral and antibacterial lipids in human milk and infant formula feeds. *Arch. Dis. Child.* **65,** 861–864

7 Thormar, H., Isaacs, C.E., Brown, H.R., Barshatzky, M.R., and Pessolano, T. (1987). Inactivation of enveloped viruses and killing of cells by fatty acids and monoglycerides. *Antimicrob. Agents and Chemo.* **31,** 27–31

8 Reiner, D.S., Wang, C., and Gillin, F.D. (1986). Human milk kills Giardia lamblia by generating toxic lipolytic products. *J. Infect. Dis.* **154,** 825–838

9 Mehta, N.R., Jones, J.B., and Hamosh, M. (1982). Lipases in preterm human milk: Ontogeny and physiologic significance. *J. Pediatr. Gastroenterol. Nutr.* **1,** 317–326

10 Committee on Food Chemicals Codex (1986). Fats and related substances. In *Food Chemicals Codex,* p. 82, National Academy Press, Washington, DC, USA

11 Reed, L.J. and Muench, M. (1938). A simple method of estimating 50 percent end points. *Am. J. Hyg.* **27,** 493–497

12 Blackberg, L. and Hernell, O. (1981). The bile-salt-stimulated lipase in human milk. Purification and characterization. *Eur. J. Biochem.* **116,** 221–225

13 Hernell, O. and Olivecrona, T. (1974). Human milk lipases. I. Serum-stimulated lipase. *J. Lipid Res.* **15,** 367–374

14 Hamosh, M., Bitman, J., Liao, T.H., Mehta, N.R., Buczek, R.J., Wood, D.L., Grylack, L.J., and Hamosh, P. (1989). Gastric lipolysis and fat absorption in preterm infants: effect of medium-chain triglyceride or long-chain triglyceride-containing formulas. *Pediatrics* **83,** 86–92

15 Silber, G.H., Hachey, D.L., Schamler, R.J., and Garza, C. (1988). Manipulation of maternal diet to alter fatty acid composition of human milk intended for premature infants. *Am. J. Clin. Nutr.* **47,** 810–814

16 Ross, A.C., Davila, M.E., and Cleary, M.P. (1985). Fatty acids and retinyl esters of rat milk: effects of diet and duration of lactation. *J. Nutr.* **115,** 1488–1497

17 Isaacs, C.E. (1990). The role of milk-derived anti-microbial lipids as anti-viral and anti-bacterial agents. (in press)

18 Kabara, J.J. (1978). Fatty acids and derivatives as antimicrobial agents—a review. In *The Pharmacological Effect of Lipids,* (J.J. Kabara, ed.), p. 1–14, The American Oil Chemists Society, Champaign, IL, USA

19 Das, S., Reiner, D.S., Zenian, J., Hogan, D.L., Koss, M.A., Wang, C., and Gillin, F.D. (1988). Killing of Giardia lamblia trophozoites by human intestinal fluid in vitro. *J. Infect. Dis.* **157,** 1257–1260

20 Coonrod, J.D. (1987). Role of surfactant free fatty acids in antimicrobial defenses. *Eur. J. Respir. Dis.* **71,** 209–214

21 Miller, S.J., Aly, R., Shinefeld, H.R., and Elias, P.M. (1988). In vitro and in vivo antistaphylococcal activity of human stratum corneum lipids. *Arch. Dermatol.* **124,** 209–215

22 Boesman-Finkelstein, M. and Finkelstein, R.A. (1985). Antimicrobial effects of human milk: inhibitory activity on enteric pathogens. *FEMS Microbiol. Lett.* **27,** 167–174