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Antimicrobial activity of lipids added to human milk, infant formula, and bovine milk

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Lipids previously shown to have antiviral and antibacterial activity in buffers were added to human milk, bovine milk, and infant formulas to determine whether increased protection from infection could be provided to infants as part of their diet. Fatty acids and monoglycerides with chain lengths varying from 8 to 12 carbons were found to be more strongly antiviral and antibacterial when added to milk and formula than long chain monoglycerides. Lipids added to milk and formula inactivated a number of pathogens including respiratory syncytial virus (RSV), herpes simplex virus type 1 (HSV-1), Haemophilus influenzae, *and Group B streptococcus. The results presented in this study suggest that increased protection from infection may be provided to infants at mucosal surfaces, prior to the digestion of milk and formula triglycerides, by the addition of antimicrobial medium chain monoglycerides to an infant's diet.* (J. Nutr. Biochem. 6:362–366, 1995.)

Keywords: lipids; viruses; bacteria; milk; formula

Introduction

Respiratory syncytial virus (RSV) has been shown to be a major pediatric viral respiratory tract pathogen world wide.[1] In the first 6 months of life, infants infected with RSV can develop serious bronchiolitis or pneumonia and during the peak period of an epidemic of lower respiratory tract disease RSV may be isolated from as many as 89% of young children admitted to a hospital.

Bacteria can also cause respiratory infections in infants. *Haemophilus influenzae*, a gram negative bacterium, is spread by airborne droplets and contact with secretions and as a result of its spread in the respiratory tract can cause otitis media, sinusitis, conjunctivitis, and bronchopneumonia. Group B streptococcus (gram positive) has been implicated in disease outbreaks spread nosocomially among hospitalized premature infants.[2,3]

Protecting infants against infection by adding food substances with anti-infective activity to their diet would be a low risk way of reducing the incidence and severity of respiratory infections. Previous studies have shown that triglycerides present in human milk and infant formulas produce antimicrobial fatty acids and monoglycerides following treatment in vitro with lipases.[4] These antimicrobial lipids inactivate enveloped viruses, bacteria, fungi, and protozoa.[5–8]

Milk- and infant formula–derived lipids are released in vivo from triglycerides primarily in the stomach and proximal small intestine. The resulting lipid-dependent antiviral activity in the stomach contents of suckling human infants decreases viral titers by ≥ 6.0 $\log_{10}$[9] in vitro. Antiviral activity is potent since diluting stomach contents up to 40-fold still produces a decrease in the titer of enveloped viruses of 3 $\log_{10}$.[10]

Lipid-dependent antimicrobial activity produced in human and bovine milks and infant formula is due to the release of long-chain unsaturated fatty acids, e.g., oleic

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acid, and medium-chain saturated fatty acids, e.g., lauric acid and their monoglycerides. The present study was designed to determine whether banked human milk, bovine milk, and most importantly infant formula, which lacks endogenous protective factors, can be made virucidal and bacteriacidal at the time of ingestion by the addition of antimicrobial lipids.

Methods and materials

Cell cultures

Vero cells (African green monkey kidney cell line; American Type Culture Collection, Rockville, MD USA) were grown in Eagle Basal medium (BME) (GIBCO Laboratories, Grand Island, NY USA) with 10% inactivated fetal bovine serum (FBS, Gemini Broproducts, Calabasas, CA USA). The maintenance medium (MM) for Vero cells was BME with 2% FBS. Gentamicin (0.1%) was added to all media.

Viruses

Herpes simplex virus type 1 (HSV-1), vesicular stomatitis virus (VSV), and respiratory syncytial virus (RSV) were obtained from the American Type Culture Collection. HSV, RSV, and VSV were grown in Vero cells. Infectious culture fluid was clarified by centrifugation at 3500 rpm for 10 min using a table-top Sorvall GLC-2B centrifuge. The pellet was discarded and the virus containing supernatant was titered on Vero cells.

Bacteria

H. influenzae and Group B streptococcus were obtained from the Microbiology Laboratory of the Consolidated Clinical Laboratories (N.Y.S. Institute for Basic Research, Staten Island, NY USA). *H. influenzae* was grown on chocolate agar plates and Group B Streptococcus on sheep blood agar plates (Scott Laboratories, Fiskeville, RI USA).

Milk and formula

Human milk samples were collected under sterile conditions 1 to 5 months postpartum and kept frozen at −86°C until use in experiments. Bovine milk was obtained from a local dairy and frozen at −86°C until needed. Infant formula was purchased from a local supplier. A vegetable oil blend (45% palm olein, 20% soy, 20% coconut, and 15% high oleic sunflower oils) was used as the fat source in the routine cow milk-based infant formula. Also the liquid formula contained <1% wet weight of mono- and diglycerides and soy lecithin as emulsifying agents. Monounsaturated fatty acids and linoleic acid accounted for 38.1% and 17.2%, respectively, of the total fat content.

Lipids

1-0-octyl-sn-glycerol (octylglycerol), an eight carbon 1-monoglyceride ether, was purchased from Deva Biotech (Hatboro, PA USA). All other fatty acids and 1-monoglyceride esters were purchased from Sigma Chemical Co. (St. Louis, MO USA). Lipids were dissolved in dimethyl sulfoxide (DMSO) (100 mg/mL) and diluted to the desired concentration. The final DMSO concentration in milk and formula was always 2%, which did not interfere with cell or virus viability.

Rationale for using ether lipids

The ether linkage is not cleaved by lipases. Enzymes capable of cleaving ether linkages are found primarily in the intestine and liver and have not been found in human milk. Monoglyceride ethers are, therefore, potentially more stable in the infant's digestive tract than the comparable esters that will be hydrolyzed by lipases in milk or produced by the infant. Ether lipids should remain antimicrobial for a longer period of time than monoglycerides with ester linkages.

Virus titration

Viruses were titrated by inoculation of 10-fold dilutions into Vero cells in 96-well microtiter tissue culture plates (Becton Dickinson Labware, Lincoln Park, NJ USA). A virus dilution (0.1 ml) in MM was inoculated into each well with three wells per dilution. The plates were kept for 3 to 7 days, depending upon the virus, and examined daily for cytopathic effect. Virus titers were calculated by the method of Reed and Muench.[11]

Assay of antiviral activity

Virus at approximately 10^5 to 10^6 50% tissue culture infective doses ($TCID_{50}$s) for HSV-1 and 10^3 $TCID_{50}$s for RSV was incubated with the appropriate lipid in human milk, bovine milk, or infant formula for 30 min at 37°C unless otherwise indicated. Virus without added lipid was used as a control. After incubation, the infectivity of each mixture was titrated by the serial dilution endpoint method. Dilutions (10-fold) were made in MM. The 10^{-2} to 10^{-5} dilutions were inoculated into monolayers of Vero cells and the virus titers were determined as described earlier. The difference between the titer ($\log_{10}$) of the control virus and the titers of lipid-virus mixtures, i.e., the reduction of virus titer, was used as a measure of antiviral activity. It was not possible to test lipid-virus mixtures in lower dilutions (10^{-1} or undiluted) because they were toxic to cell cultures. Assuming that the 10^{-1} dilution contained infectious virus, the highest possible titer of the lipid virus mixtures in $10^{1.5}$ $TCID_{50}$, and the reduction of virus titer ($\log_{10}$) would equal 4.0 if the initial virus titer was $10^{5.5}$ (5.5 minus 1.5). If the titers of the mixtures were less than $10^{1.5}$, the reduction of titer would be greater than 4.0.

Assay of antibacterial activity

H. influenzae and Group B streptococcus were adjusted to concentrations of 10^4/ml to 10^5/ml in milk or formula and the lipid to be tested was added. After incubation for 1 hr at 37°C, the bacteria plus milk or formula mixtures were diluted in 10-fold increments, plated on chocolate agar or sheep blood agar, and incubated at 37°C until bacterial colonies could be counted. Bacterial inactivation was determined by subtracting the number of colonies on plates of lipid-treated bacteria from the number on control plates.

Results

Fatty acids and monoglycerides, previously shown to have antiviral activity in buffers, were tested against several enveloped viruses in human and bovine milk and infant formula. VSV was inactivated by octylglycerol and monocaprin following their addition to human milk (*Table 1*). Viral inactivation was dependent upon the lipid concentration and the duration of incubation. Octylglycerol was more antiviral than monocaprin on a concentration basis during the same incubation period. When the incubation period was doubled, monocaprin inactivated comparable levels of virus.

Table 1 Inactivation of VSV by lipids added to human milk

Lipid*	Concentration (mM)	Incubation (min)†	Reduction in viral titer (log_{10})‡
Octylglycerol	7.5	5	≥4.0§
	15	2	3.5§
	20	2	≥4.0§
Monocaprin	7.5	5	2.25§
	7.5	10	≥4.25‖
	20	2	2.0§
	20	5	≥4.0§
Lauric acid	5	10	≥4.25‖

*Octylglycerol is an eight carbon monoglyceride ether. Monocaprin is a 10 carbon monoglyceride ester. Lauric acid is a 12 carbon fatty acid.
†All incubations were at 37°C.
‡Controls with 2% DMSO did not show viral inactivation or cell toxicity.
§Human milk alone reduced the viral titer 0.5 log_{10}.
‖Human milk alone reduced the viral titer 1.0 log_{10}.

Table 3 HSV-1 inactivation in infant formula by added fatty acids and monoglycerides

Lipid*	Concentration (mM)	Log_{10} reduction in HSV-1 titer
Monocaprin	4.5	1.5
	7.5	1.75
	15	≥3.75
	22.5	4.0
	45	4.0
Monocaprylin	4.5	0
	7.5	≥3.75
	15	≥3.75
	45	≥4.0
Caprylic acid	15	0.25
	30	0.25
	60	1.0
Capric acid	30†	≥3.75

*Monocaprylin is an eight carbon monoglyceride ester. Caprylic acid and capric acid are 8 and 10 carbon fatty acids, respectively.
†Lowest concentration to reduce viral titers.

Lauric acid had antiviral activity similar to the monoglycerides tested.

Fatty acids and monoglycerides also inactivated VSV in bovine milk (*Table 2*). Lauric acid by itself and mixtures of lauric acid with either monocaprin or octylglycerol inactivated all of the virus at a total concentration of 5 mM. Octylglycerol and monocaprin required concentrations of 15 and 7.5 mM respectively, to completely inactivate VSV in the same 10 min time period.

Monoglycerides more effectively inactivated HSV-1 on a concentration basis (mM) than the corresponding fatty acid when added to infant formula (*Table 3*). Monocaprin (10:0) and monocaprylin (8:0) at concentrations of 15 mM reduced the HSV-1 titer in formula from almost 180,000 IU/ml to <100/ml. Capric acid produced the same virus titer decrease as the monoglycerides but only at a concentration 2-fold higher (30 mM). Caprylic acid even at a concentration of 60 mM only reduced HSV-1 titers by 1 log_{10}.

RSV was inactivated by monocaprin at 22.5 mM and monocaprylin and monoolein at 45 mM in infant formula (*Table 4*). Other monoglycerides with chain lengths ranging from 12 to 18 carbons (both saturated and unsaturated) did not inactivate the virus.

Table 2 Inactivation of VSV by lipids added to bovine milk*

Lipid	Concentration (mM)	Reduction in viral titer (log_{10})†
Octylglycerol	7.5‡	2.25
	15	≥3.25
Monocaprin	7.5‡	≥3.25
Lauric acid	5	≥3.25
Octylglycerol-lauric acid	2.5 + 2.5	≥3.25
Monocaprin-lauric acid	2.5 + 2.5	≥3.25

*All incubations were at 37°C for 10 min.
†Bovine milk by itself reduced the viral titer 1.25 log_{10}.
‡Lowest concentration to reduce viral titers.

Monoglycerides with chain lengths varying from 8 to 18 carbons were added to infant formula and tested against major infant bacterial pathogens. Medium-chain saturated and long-chain unsaturated monoglycerides inactivated *H. influenzae* (*Table 5*). The 8 and 10 carbon monoglycerides were maximally effective at a concentration of 4.5 mM, whereas monolinolein (18:2) and monolinolenin (18:3) inactivated the bacterium at the higher concentrations of 45mM and 22.5 mM, respectively.

Inactivation (>4.0 log_{10}) of Group B streptococcus was found at a concentration of 4.5 mM monocaprin added to infant formula (*Table 6*). However, none of the other monoglycerides tested reduced the bacterial concentration when they were added at 4.5 mM. Raising the monoglyceride concentrations to 22.5 mM resulted in a change from no inactivation to maximum bacterial inactivation with monocaprylin (8:0) and monopalmitoleic (16:1) acid (16:1). A monolinolein (18:2) concentration of 45 mM was required to reduce the Group B streptococcus concentration by ≥4.0

Table 4 Inactivation of RSV by monoglycerides added to infant formula

Monoglyceride*	Monoglyceride concentration (mM)		
	4.5†‡	22.5	45
8:0	0.5	0.5	≥1.8
10:0	1.3	≥2.0	≥2.0
12:0	0.5	0	1.3
14:0	0	0	1.0
16:1	0	0	0.8
18:1	0	0	2.0
18:2	0	0	1.0
18:3	0	0	0.8

*The numbers refer to the chain length and number of double bonds of the fatty acids, e.g., oleic acid is (18:1).
†All numbers are the reduction of virus titer (log_{10}).
‡The RSV control titer was 3.5 which is 2 logs lower than that used with HSV-1.

Table 5 Inactivation of *H. influenzae* by monoglycerides added to infant formula

Monoglyceride	Monoglyceride concentration (mM)		
	4.5	22.5	45
8:0	≥4.0*	≥4.0	≥4.0
10:0	≥4.0	≥4.0	≥4.0
12:0	0	≥4.0	≥4.0
14:0	0	0	0
16:1	0	0	0
18:1	0	0	0
18:2	0	0	3.0
18:3	0	3.0	3.0

*All numbers are the reduction of bacterial concentration ($\log_{10}$).

$\log_{10}$. Monolaurin (12:0), monomyristin (14:0), monooleic (18:1), and monolinolein (18:2) did not reduce the colony forming ability of the bacteria.

Discussion

The lipid fraction taken from the stomach contents of infants fed milk or infant formula inactivates enveloped viruses as well as grain positive and gram negative bacteria.[9] This lipid-dependent antimicrobial activity can be duplicated using purified long-chain unsaturated and medium-chain saturated fatty acids whether free or linked to glycerol as a primary ester.[5,12] In human and bovine milks, oleic acid is the primary antimicrobial fatty acid released upon triglyceride hydrolysis.[13] In some infant formulas, lauric acid makes up as much as 25% of the fatty acid content, but these high lauric acid formulas also contain over 40% combined oleic and linoleic acids.[14]

Milk contains lipases that can hydrolyze monoglyceride ester linkages to glycerol and free fatty acid. Our data indicate that free fatty acids less than 12 carbons in chain length added to milk and infant formula are less antiviral than the corresponding monoglyceride. Attaching octanoic acid to a glycerol backbone by an ether linkage, i.e., octylglycerol, produces compounds that are as antimicrobial as naturally occurring ester linked monoglycerides, but which are not susceptible to lipolysis by digestive lipases found in milk or produced by the infant.

Results from previous studies, showing that certain long-chain unsaturated fatty acids have strong antiviral activity,[5] suggested that adding these fatty acids and their monoglycerides to milk or infant formula might be an effective way to provide increased antimicrobial protection to infants from the time of ingestion. In this study, monoglycerides with chain lengths greater than 12 carbons were less effective than medium chain lipids (*Tables 4, 5,* and *6*) when added to human and bovine milks and infant formula (results with long-chain fatty acids in human and bovine milk were not shown). The greater efficacy of medium-chain fatty acids in complex media may result not only from the greater aqueous solubility of fatty acids as their chain length decreases but also from the fact that longer chain lipids may bind more readily to milk and formula proteins, e.g., albumin, and thus become less available for interactions with microbes.[15,16]

Lipid mixtures can be customized for addition to milk and formula to inactive specific viral and bacterial pathogens. For example, it has been shown that some gram negative bacteria, e.g., *E. coli,* are more strongly inactivated by short-chain fatty acids than medium- or long-chain fatty acids.[17] If the targeted pathogens in a particular geographic area are gram negative bacteria, mixtures can be made of short-chain and medium-chain fatty acids to target gram negative bacteria as well as enveloped viruses and gram positive bacteria.

A number of the pathogens inactivated in this study by the addition of lipids to milk and formula are particular problems for infants, especially premature infants. There is no vaccine available for RSV or HSV-1 while *H. influenzae* vaccination is for infants 18 months and older.[1,2] Recent studies indicate that RSV immune globulin can be given to high-risk infants[18] but additional protective measures would be valuable since some infants have adverse reactions to the immune globulin.

RSV is a major cause of nosocomial infection and is a particular problem to premature infants. In order for a vaccine to be useful, it would have to stimulate resistance by the age of 2 months which is the peak age of infection. Seventy percent of infants requiring hospitalization for RSV are under 6 months of age.[1] This virus is spread by close contact with large droplets and by self-inoculation after touching contaminated skin or objects, but not by small particle aerosols.[21] During infant feeding, the milk or formula, in addition to being ingested, comes into contact with the infant's lips and other parts of the face and can potentially reduce the RSV inoculum size. Routinely feeding infants a diet that contains antiviral lipids may help to reduce the incidence of RSV infection especially in a nursery.

Bacterial pathogens such as *H. influenzae* and group B streptococcus were shown to be inactivated in this study. *H. influenzae* spreads by invading the submucosa of the nasopharynx. A recent study[22] shows that secretory IgA in human milk can protect against nasopharyngeal colonization by *H. influenzae*. This provides evidence that antibacterial agents ingested in the diet can potentially protect against *H. influenzae* infection. Infant formulas lack protective antibodies. Antiviral and antibacterial lipids may potentially provide an alternative mechanism for reducing *H. influenzae* infection in infants. Since antimicrobial lipids

Table 6 Inactivation of Group B streptococcus by monoglycerides added to infant formula

Monoglyceride	Monoglyceride concentration (mM)		
	4.5	22.5	45
8:0	0[1]	≥4.0	≥4.0
10:0	≥4.0	≥4.0	≥4.0
12:0	0	0	0
14:0	0	0	0
16:1	0	≥4 0	≥4.0
18:1	0	0	0
18:2	0	0	0
18:3	0	0	≥4.0

*All numbers are the reduction of bacterial concentration ($\log_{10}$).

function in vivo at mucosal surfaces,[19,20] providing antimicrobial lipids to infants during feeding would supplement a protective mechanism already at mucosal surfaces.

Group B streptococcus infection is a concern for both term and preterm infants.[3] The bacterium can present as either early onset or late onset disease. Early onset is the development of symptoms during the first 5 days of life. Premature infants are affected significantly more frequently than term infants by early infection. Late onset infection afflicts premature infants occasionally but primarily affects term infants from 7 days to 3 months of age. Antimicrobial lipids could be used to provide additional protection to infants at risk for the late onset disease.

Currently, monoglycerides are added in small amounts (less than 1% wet weight) to liquid infant formulas as emulsifying agents. Thus, it is technically feasible to add monoglycerides to infant formulas. The feasibility issues of adding higher levels of monoglycerides to provide antimicrobial activity are ingredient costs and the impact on taste. Alterations in taste usually result from the development of rancidity resulting from lipid hydrolysis and/or oxidation. The use of monoglyceride ethers with saturated fatty acids, e.g., octylglycerol, should eliminate this problem. Clinical testing will have to be done to definitively answer this question.

The results from this study suggest that medium-chain fatty acids or monoglycerides added to infant formulas as well as human and bovine milks provide antimicrobial protection against viral and bacterial pathogens prior to digestion. This includes protection provided by preventing or reducing the growth of microbial contaminants in the infant's food prior to ingestion. Diets containing antimicrobial factors may be of particular value to population groups at increased risk for infection, such as premature infants in hospital nurseries.

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