Membrane-Disruptive Effect of Human Milk: Inactivation of Enveloped Viruses

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Fresh human milk treated with antibody to secretory IgA had no effect on viral infectivity but became antiviral after 1 hr in the stomachs of suckling infants. Antiviral activity also appeared in fresh milk stored at 4 C for at least two days. The antiviral activity, which reduced titers of virus by as much as 10,000-fold, only affected enveloped viruses and was localized in the milk lipid fraction. Its appearance in stored milk was apparently due to fatty acids released by the activity of milk lipases, particularly lipoprotein lipase. Antiviral activity in the infants' stomach, however, most likely resulted from the activity of gastric and lingual lipases on milk triglycerides and caused the release of antiviral fatty acids. Milk and stomach contents that were antiviral also lysed cultured cells by disruption of their plasma membrane. Cell lysis was also caused by purified linoleic acid, which is a normal constituent of human milk triglycerides.

Breast-fed infants have been reported to have a lower incidence of gastrointestinal infections than do infants fed formulas or cow's milk [1-3]. Although the resistance to infection has largely been attributed to milk-specific immunoglobulins, primarily secretory IgA (SIgA), other studies show that breast milk also contains nonspecific antimicrobial factors [4]. Thus, antiviral activity that inactivated only enveloped viruses has been reported in some stored human milks. This antiviral activity was localized in the lipid fraction, was apparently dependent on free unsaturated fatty acids and monoglycerides, and correlated with the level of milk lipase [5, 6]. However, not all milk specimens with high levels of lipase were antiviral, and the exact role of lipases was not well established. In addition, the physiological significance

of these findings remains to be determined because it is not known whether milk acquires antiviral activity *in vivo*, i.e., in the gastrointestinal tract of infants.

Similar nonspecific lipid-associated activity against *Giardia lamblia* and other parasitic protozoa has been found in stored, SIgA-depleted human milk [7, 8]. It was concluded initially that the giardicidal activity results from the direct action of human milk bile salt-stimulated lipase (BSSL) [7, 8]. However, more recent work suggests that an additional type of killing activity against *Giardia* was present in human milk and was the result of free fatty acids released during storage of milk [9]. Whether milk lipases are necessary to elicit a milk lipid-dependent antimicrobial effect *in vivo* or whether, as suggested by Gillin et al. [8], milk lipase is directly antimicrobial has not been established.

In the present work we studied the appearance of antiviral activity in the lipid fraction of fresh and stored human milk. We also examined the antiviral activity of human milk retrieved from the gastrointestinal tract of suckling human infants.

Materials and Methods

Samples of milk and stomach contents. Samples of human milk were collected at five days to 10 months postpartum and tested against human and animal viruses on the same day, after storage at -80 C for up to one year or after various intervals at 4 C.

Samples of stomach contents from infants fed hu-

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Parental informed consent was obtained before samples were taken, and guidelines for human experimentation as required by the U. S. Department of Health and Human Services, the New York State Institute for Basic Research, and Columbia University College of Physicians and Surgeons were followed.

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man milk with a nasogastric tube were obtained. Milk was from the infant's mother, a donor, or both. Whole milk, previously frozen at -80°C , was fed to infants every 3 hr by nasogastric tube, and samples of stomach contents were taken at 1 and 3 hr after feeding. Some human milk samples were pasteurized and then supplemented with protein and calcium before feeding. All samples of human milk and stomach contents were supplied by Dr. William C. Heird (Department of Pediatrics, College of Physicians and Surgeons, Columbia University, New York). Fresh and unpasteurized cow's milk was supplied by Dr. Mark Eppler (American Cyanamid, Princeton, NJ). Purified lipoprotein lipase (LPL) was purchased from Sigma (St. Louis).

Cell cultures. Vero cells (African green monkey kidney cell line; Flow Laboratories, McLean, Va) were grown in Eagle's basal medium (Gibco, Grand Island, NY) with 10% inactivated fetal calf serum (Gibco) and maintained in Eagle's basal medium with 2% fetal calf serum. Sheep fibroblast cultures were obtained from the choroid plexus of lamb brains and grown in Eagle's basal medium with 15% lamb serum and maintained in Eagle's basal medium with 2% lamb serum. Gentamicin (0.1%) was added to all media.

Viruses. Measles virus strain Edmonston, herpes simplex virus type 1 strain MacIntyre, vesicular stomatitis virus (VSV) strain Indiana, poliovirus type 1 strain Chat, and vaccinia virus strain WR were all grown in Vero cells. For certain experiments, herpes simplex virus grown in sheep fibroblasts was also used. All strains of virus were obtained from the American Type Culture Collection (Rockville, Md) except poliovirus, which was obtained from Dr. R. I. Carp (New York State Institute for Basic Research, Staten Island).

Titration of virus. Viral infectivity was titrated by inoculation of 10-fold dilutions into Vero cell cultures in 96-well microtiter tissue culture plates (Falcon Plastics, Oxnard, Calif). One-tenth milliliter of a dilution of virus in maintenance medium was inoculated into each well, four wells per dilution. The plates were kept at 37°C for two to 10 days, depending on the virus, and examined daily for CPE. Titers of virus were calculated by the method of Reed and Muench [10].

Assay of antiviral activity. Milk samples were diluted 1:5 in maintenance medium containing $\sim 10^5$ TCID₅₀ of virus and incubated at 37°C for 30 min. Virus mixed with maintenance medium instead of

milk was used as a control. After incubation the infectivity of each mixture of milk and virus and each viral control was titrated, and the difference (in log units) between the titer of the viral control and the titer of virus incubated with milk, i.e., reduction in titer of virus, was used as a measure of antiviral activity. Milk samples with antiviral activity were cytotoxic at a dilution of ≤ 10 -fold. Mixtures of milk and virus were therefore diluted ≥ 100 -fold so that the milk was no longer toxic for the Vero cell cultures used in the assay for virus.

Milk fractionation. Milk was stored either unfractionated or separated on day 1 into cream and supernatant fractions by centrifugation at 17,000 g for 60 min at 4°C . The lipid fraction was on top, and the supernatant was directly below it. Unfractionated milk samples were stored at 4°C for four to five days and then separated into cream and supernatant by centrifugation.

Enzyme assays. BSSL and LPL were assayed as previously described [11]. Buffers and chemicals for the assays were purchased from Sigma. A substrate emulsion for both enzymes was prepared with glyceryl (9, 10) (*n*)-[^3H]oleate (C Triolein; New England Nuclear, Boston) with two 30-sec bursts from a Polytron® homogenizer (Brinkmann, Westbury, NY) at a setting of 10. The assay mixture for BSSL was 60 mM Tris-HCl (pH 8.6), 2.8% bovine serum albumin, 1.7 mM triglyceride, and 12 mM sodium taurocholate in a total volume of 200 μl . The LPL assay mix, in a final volume of 200 μl , was 200 mM Tris-HCl (pH 8.6), 5% bovine serum albumin, 3 mM triglyceride, and 0.25 U of heparin. Incubation was at 37°C for 15 min with BSSL and 20 min with LPL. In both instances the reaction was stopped by addition of 3.25 ml of a mixture of methanol-chloroform-heptane (1.41:1.25:1.0 vol/vol/vol). Free fatty acids were extracted with 1.05 ml of 0.05 M potassium carbonate, pH 10. The radioactivity in the aqueous phase was measured with a liquid scintillation spectrometer (Tri-Carb; Packard Instruments Co., Downer's Grove, Ill).

Preparation of cells for electron microscopy. Cell cultures were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer. They were then rinsed in 0.1 M cacodylate buffer and postfixed with 2% osmium tetroxide in 0.1 M cacodylate buffer. The cells were dehydrated through a graded series of ethanol, critical-point dried, and sputter coated with 105 Å of gold. Each preparation was viewed and evaluated with a scanning electron microscope at 20 kV.

Table 1. Reduction of viral infectivity titers by human milk.

Milk	Enveloped			Nonenveloped	
	Measles virus	Herpes simplex virus	VSV	Vaccinia virus	Poliovirus
Fresh	0	0	0	0	0
Stored at -80 C	0	0	0	0	0
Stored at 4 C for 9 days	≥3.0	≥3.0	≥3.5	0	0

NOTE. Milk was collected from 10 women five days to 10 months postpartum. Each sample was divided into three parts and assayed for antiviral activity fresh, after storage at -80 C for at least two months, or after storage at 4 C for nine days. Samples were incubated with a 1:10 dilution of antibody to human colostral IgA (Dako Corp., Santa Barbara, Calif) for 2 hr at 23 C directly before assay for viral infectivity. Data are log units.

Results

Fresh or deep-frozen (-80 C) milk did not exhibit antiviral activity after pretreatment with antibody to human colostral SIgA. However, aliquots of milk samples similarly treated with antibody but stored at 4 C for various intervals (two to seven days) inactivated enveloped RNA and DNA viruses (measles virus, VSV, and herpes simplex virus) but not non-enveloped viruses (poliovirus and vaccinia virus; table 1). Thus, milk stored at 4 C diluted fivefold and mixed with enveloped viruses caused a >3 log reduction in titer of virus. Because none of the human milk samples had antibodies to VSV, an enveloped animal virus, all further studies were done with VSV to eliminate the need for pretreatment with antibody to human IgA.

The antiviral activity that appeared in milk during storage at 4 C and in the stomach contents obtained from an infant 1 hr after feeding was local-

ized in the cream fraction (table 2). However, the supernatant was needed to activate the lipid because separation of the two fractions on the day the milk was collected prevented appearance of antiviral activity in the cream fraction when it was stored separately at 4 C.

All of the human milk samples tested had sufficient BSSL to release fatty acids from human milk triglycerides in the presence of bile salts, but the samples that became antiviral were those with high levels of another milk lipase, LPL (table 3). When purified LPL was added to samples 5-7 (table 3), they acquired the same level of antiviral activity as human samples with endogenous LPL activity. That BSSL is not required for this process is also indicated by the results with fresh unpasteurized cow's milk, which contains LPL but not BSSL and becomes antiviral after storage (table 3).

To answer the question of whether milk becomes antiviral *in vivo*, we diluted stomach contents, obtained from infants fed unpasteurized human milk via a nasogastric tube, 1:5 in medium at neutral pH and tested them against VSV (table 4). At 1 hr after feeding, all samples had antiviral activity, which was localized in the lipid fraction, just as it was in stored milk (table 2). That this antiviral activity was derived from the milk and not secreted by the stomach was suggested by the observations that 3-hr stomach contents samples from some infants were no longer antiviral and that 1- and 3-hr samples from infants fed pasteurized human milk did not inactivate viruses (table 4). None of the milk samples fed to infants inactivated the virus, with the exception of sample 3, which became even more antiviral after 1 hr in the infant's stomach. The variability of antiviral activity at 3 hr reflects various rates of digestion by the different infants because gastric emptying is usually complete within 3 hr [12].

Antiviral milk samples lysed the cell cultures, ei-

Table 2. Reduction in titer of VSV by fractions of milk and stomach contents.

Sample	Unfractionated sample	Separation before storage		Separation after storage	
		Cream	Supernatant	Cream	Supernatant
Human milk 1*	≥4.5	0	0	≥4.5	0
Human milk 2*	≥3.5	0	0	≥3.5	0
Human stomach contents†	≥4.0	≥3.5	0	ND	ND

NOTE. ND = not done.

* Assayed after storage at 4 C for nine days.

† Obtained 1 hr after feeding.

ther Vero cells or sheep fibroblasts, used for assay of virus and viral replication, whereas fresh milk did not visibly affect these cells. Scanning electron microscopy of Vero cells incubated with a fivefold dilution of antiviral stomach contents from two infants fed fresh human milk over four weeks showed disintegration of the cellular membrane (figure 1, upper right). Incubation with linoleic acid (Sigma), a common fatty acid of milk that has been found to inactivate enveloped viruses [6], affected the cell membrane in the same way (figure 1, lower right). Cells incubated with fresh milk or with stomach contents from two infants fed pasteurized human milk over two weeks (table 4) showed normal unaffected membranes (figure 1, upper and lower left), identical to those of cells incubated in medium (data not shown). Because the lipid bilayer of viral envelopes is derived from the host cell membrane, these results indicate that the inactivation of enveloped viruses by milk lipids reflects the more general phenomenon of cell membrane disruption. The effect of milk on lipid bilayers is probably due to the release by lipases of linoleic and other fatty acids present in milk triglycerides.

Discussion

The appearance of lipid-dependent antimicrobial activity in stored human milk is probably the result of milk-handling procedures that disrupt the milk fat globule membrane and make milk triglycerides

Table 3. Relation of levels of human milk BSSL and LPL with reductions in titers of virus.

Sample	BSSL ($\mu\text{mol/ml per min}$)	LPL (nmol/ml per min)	Reduction (log units) in VSV titer
Human			
1	9.83	508	≥ 3.5
2	7.63	209	≥ 3.5
3	1.55	159	≥ 3.5
4	4.98	336	≥ 3.0
5	9.51	20	0
5*	9.51	220	≥ 3.5
6	5.42	0	0
6*	5.42	200	≥ 3.5
7	5.87	2	0
7*	5.87	202	≥ 3.5
Bovine	. . .	338	≥ 4.0

NOTE. Human milk samples were from seven different women.

* Samples with purified LPL added to a concentration of 200 nmol/ml per min.

Table 4. Reduction in titer of VSV by stomach contents from milk-fed human infants.

Sample	Milk	Reduction (log units) in VSV titer by stomach contents after feeding	
		1 hr	3 hr
1	0	≥ 4.0	3.0
2	0	≥ 4.0	0
3	2.0	≥ 4.0	≥ 4.0
4	0	≥ 4.0	0
5	0	≥ 4.0	≥ 4.0
6	0	0	0
7	0	0	0

NOTE. Samples 1–5 are fresh whole human milk, and samples 6 and 7 are pasteurized human milk. Samples 1–4 were taken from the same infant on four successive weeks; samples 5–7 were taken from three additional infants.

available to LPL. The suckling infant's stomach has both gastric lipase and lingual lipase activities [13], which can release fatty acids from milk triglycerides. It is likely that these enzymes are responsible for the release of milk antiviral lipids in the infant's stomach because there are no bile salts to activate BSSL and because LPL is inactivated in the stomach [14]. The enzymatic process of fatty acid release in the stomach is much more rapid than in stored milk, in which antiviral activity did not appear until after at least two days at 4 C. The failure of pasteurized milk to become antiviral may indicate that the concentrations of some milk lipids are decreased by the process. For example, the concentrations of linoleic and linolenic acid in human milk are decreased by freezing and thawing and by heating [15].

Lipid-dependent antiviral activity is one of many protective factors present in human milk [16]. The decreased incidence of gastrointestinal infections found in milk-fed human infants [1–3] undoubtedly results from the activity of all of the protective factors that occur in milk, both immunoglobulins and nonspecific factors. An infant fed cow's milk would get lipid-dependent antimicrobial activity but not the appropriate immunoglobulins and would be at greater risk for infection than would a breast-fed infant. Also, the presence of multiple protective factors in human milk suggests that it is not necessary for any one factor to have a broad spectrum of antimicrobial activity.

Antimicrobial fatty acids in the gastrointestinal tract should expand the range of protection against

Figure 1. Scanning electron micrographs show the effect of human milk on cell cultures (bars, 1 μ m). Monolayers of Vero cells were incubated at 37 C for 30 min with (*upper left*) fresh milk, (*upper right*) 1-hr stomach contents of an infant fed fresh milk, (*lower left*) 1-hr stomach contents of an infant fed processed milk, and (*lower right*) linoleic acid in Eagle's basal medium, 1 mg/ml. Milk and stomach contents samples were diluted 1:5 in Eagle's basal medium. The left panels show intact cell membranes with microvilli, whereas in the right panels the cell membranes are partly or completely disintegrated.

intestinal pathogens provided by milk immunoglobulins, as evidenced by our observation that the type of host cell used to grow enveloped viruses does not affect inactivation of virus by milk. For example, surface antigen differences are common among different isolates of *G. lamblia* [17]. Membrane-disrupting fatty acids would be able to inactivate all isolates of *G. lamblia*, whereas SIgA in milk would only reflect the mother's previous exposure. Nonspecific protective factors are constantly present in milk and thus provide immediate protection against previously unencountered pathogens in the absence of the appropriate SIgA.

Although most known gastrointestinal viruses are nonenveloped, antiviral fatty acids can still play an important antiviral role in the intestine. Necrotizing enterocolitis in infants can be caused by a human enteric coronavirus [18]. This virus is enveloped and is thus susceptible to inactivation by antiviral fatty acids.

The results presented here show that fresh human milk is not antiviral but becomes so in the stomach of the suckling infant, apparently as the result of the release of fatty acids that can disrupt plasma membranes. Therefore there are not different classes of human milk, some of which are antiviral and others

not, as had been previously suggested on the basis of studies with stored human milk [5]. Whether there are any dietary constraints on this fatty acid-dependent mechanism has not been conclusively established. However, because more than one type of fatty acid appears capable of producing the effect [5], it is unlikely that any human milk would completely lack lipid-dependent antimicrobial activity.

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