INFECTION AND IMMUNITY, Feb. 1978, p. 395-401
0019-9567/78/0019-0395$02.00/0

Vol. 19, No. 2
Printed in U.S.A.

Use of Semliki Forest Virus to Identify Lipid-Mediated Antiviral Activity and Anti-Alphavirus Immunoglobulin A in Human Milk

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Received for publication 1 August 1977

In a simple and reliable assay system, Semliki forest virus (SFV) was used to detect the activity of antiviral factors in human milk. Fractionation of the milk showed that a heat-stable, lipid-associated activity and an immunoglobulin-associated activity were present, either singly or together, in 85% of the human milk samples tested. Cow and synthetic milk showed neither activity. Extraction of the neutral milk lipids allowed the antiviral activity to be located with the monoglyceride and free fatty acid fractions. The milk low in antiviral lipids and high in triglycerides also lacked a strong lipase activity. The immunoglobulin anti-SFV activity was shown to be due to immunoglobulin A, the major milk immunoglobulin, and appears to be directed against an alphavirus closely related to SFV, possibly Ross River virus.

It has long been recognized that breast feeding confers on infants a degree of resistance to infection not present in those fed cow or synthetic milk (6, 11, 15, 18). The exact cause of this resistance is not completely understood, though several antibacterial and antiviral factors have been detected in human milk. The antiviral factors include immunoglobulins, particularly immunoglobulin A (IgA), immunoglobulin-producing cells (15, 17), hemagglutinin inhibitors (13, 22), interferon (8), non-immunoglobulin macromolecules (16, 19), ribonuclease (3, 16), and nonspecific uncharacterized lipids (9, 10).

Although it may be true that immunoglobulin is the most significant of these (7), it is easy to envisage circumstances in which nonspecific antibacterial or antiviral factors could play an important prophylactic role—for example, in the absence of specific or cross-reacting maternal antibody.

We examined here the antiviral activity of human milk using an alphavirus (Semliki forest virus, SFV), with particular emphasis on uncovering the nature of the nonspecific antiviral lipids.

MATERIALS AND METHODS

Human milk specimens. Milk samples, both pooled and individual, were obtained from women, 9 to 25 days postpartum, through the courtesy of D. Leslie, Royal Women's Hospital, Melbourne, Australia. Samples were held at 4°C at the hospital either overnight (evening samples) or for a maximum of 3 h (morning samples) before being transported to La Trobe University, Victoria, where they were frozen and stored at −18°C.

Viruses and cells. Plaque-purified SFV, Herpes simplex virus types 1 and 2 (HSV-1, HSV-2), adenovirus type 2 (Ad-2), influenza A_0/WSN, simian adenovirus 15 (SV15), and simian virus 40 (SV40) were obtained from stocks held by the Microbiology Department, La Trobe University. Stocks of SFV (strain 25639) for experiments were prepared by infecting BHK-21 cells at a multiplicity of infection of less than 1.0 to avoid the problem of defective interfering particles. The alphaviruses, Getah (N555), Bebaru (AMM2454), Ross River (T48), and Sindbis (MRM39), were a gift of I. Marshall (Canberra, Australia). BHK-21 cells were maintained on Eagle minimal essential medium (EMEM, prepared using Autopow, Flow Laboratories, Inc., Rockville, Md.) supplemented with 100 μg of neomycin per ml and 10% calf serum (Flow Laboratories, Inc. Sydney) or 10% fetal calf serum (Commonwealth Serum Laboratories, Melbourne).

Test for antiviral activity. The virus under test was diluted in 10-fold steps in virus diluent (EMEM containing 200 μg of bovine serum albumin per ml and 100 μg of neomycin per ml). Mixtures of virus in diluent (1:1, vol/vol) and virus in milk (1:1, vol/vol) were incubated at 37°C for 60 min. Then, 0.2-ml volumes were adsorbed for 60 min onto the appropriate near-confluent cell monolayers, which were overlayed with 1% agarose (L'Industrie Biologique Francaise, S.A., Gennevilliers) in a mixture of EMEM (containing 100 μg of neomycin per ml plus 10% calf serum)-tryptose phosphate broth-phosphate-buffered saline (PBS) (6:1:3) held at 50°C. Plates were incubated at 37°C in a 5% CO_2 atmosphere for 40 h (alphaviruses on BHK cells), 96 h (HSV-1, HSV-2 on BHK cells), or 8 days (Ad-2 on KB cells, SV15 on CV-1 cells, SV40 on CV-1 cells, and influenza A_0/WSN

on CEF cells). All plates to be incubated for 8 days had a second nutrient agarose overlay on day 5. After the incubation period, all plates were flooded with 0.1% (wt/vol) crystal violet and allowed to dry at room temperature overnight. The agarose overlay was then carefully removed, leaving the monolayer intact with plaques clearly visible. In the case of influenza A_0/WSN, no plaques could be seen before staining and agarose removal. For rapid assay of anti-SFV activity, the virus was diluted to give a total of ca. 100 plaque-forming units per 0.2-ml volume added per plate. This made it unnecessary to assay at two or more virus dilutions.

Fractionation of milk. Twenty-milliliter volumes of milk were centrifuged at 10,000 rpm for 20 min at 4°C in a Sorvall RC-5 centrifuge. The cream fractions were suspended in PBS (10) to make up the original volume of milk. The skim fractions were not added to. Milk was placed in sealed tubes for boiling where required.

When further extraction of the cream fraction was required, the undiluted cream was suspended directly in 20 ml of a mixture of methanol–chloroform (1:2 vol/vol) and stirred gently at room temperature for 60 min. The extract was centrifuged at 10,000 rpm for 20 min at room temperature. The supernatant was dried by N_2 gassing and dissolved in 10 ml of chloroform. Two grams of silicic acid (Sigma Chemical Co., St. Louis, Mo.) was added per 10-ml volume, and the mixture was shaken at room temperature for 30 min. After centrifugation, the supernatants were either dried by N_2 gassing and resuspended in the original milk volume of PBS for antiviral assay or loaded directly (50 μl) onto thin-layer chromatography (TLC) silica gel plates (Merck, Darmstadt, West Germany) for separation of neutral lipids by a mixture of *n*-hexane–diethyl ether–acetic acid (90:10:1 or 60:40:2). Unsaturated lipids were detected after the plate stood for 30 s in iodine vapor. The lipids were eluted from the silica plates by scraping the portion of the plate containing the lipid into chloroform and eluting for 15 min at room temperature. The silica was removed by low-speed centrifugation, and the chloroform layer was dried. For antiviral assays, the lipid was resuspended in PBS. A negligible amount of IgA or immunoglobulin M (IgM) was found associated with any lipid fraction by use of immunodiffusion techniques on S-Partigen IgA, IgM, or immunoglobulin G (IgG) immunodiffusion plates (Boeringer-Hoescht, Frankfurt, Germany).

Phospholipids were recovered from the silicic acid by shaking it with methanol for 30 min and, after centrifugation, by treating it in a manner similar to that for the neutral lipids, except that chromatograms were developed in chloroform–methanol–acetic acid (25:15:4).

Skim milk (10 ml) was fractionated by making it 40% saturated with respect to ammonium sulfate. After standing at 4°C for 30 min, the mixture was centrifuged at 10,000 rpm for 10 min, and the pellet was collected. This protein was either dissolved in the original skim volume of PBS and assayed for antiviral activity or dissolved in 2 ml of PBS of which 100 μl was loaded onto a Sephadex G-200 chromatography column (50 by 1.8 cm) and fractionated by elution with 20 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.5. Fractions (1 ml) were collected after the elution of the void volume and tested for antiviral activity. The presence of IgA, IgG, and IgM was detected by radial immunodiffusion technique on S-Partigen IgA, IgM, or IgG immunodiffusion plates (Boeringer-Hoescht). Total milk IgA was also measured by this technique.

Assay of lipase activity in milk and milk fractions. Lipase activity was determined by allowing 10-μl volumes of milk or milk fractions to act on a 2-ml volume of substrate comprising 0.4% (vol/vol) tributyrin (glycerol tributyrate) in 100 mM $NaCl_2$, 10 mM $CaCl_2$, 50 μg of neomycin per ml, and 0.8 mg of bromothymol blue (pH 7.8) per ml. Activity was measured by timing the change in pH from 7.5 to 6.5. One unit of lipase activity was that amount of activity lowering the pH of our assay medium by 1 pH unit per min. Boiled milk was used as the control.

RESULTS

Detection of antiviral activity of human milk. We examined the effect of human milk on a variety of human and animal viruses by the plaque reduction neutralization technique to determine the nature of the antiviral factors. The effect of human milk samples on the activity of some animal and human viruses was tested (unpublished data). The lipid-enveloped viruses tested (HSV, influenza, and SFV) were all found to have their plaque-forming activity decreased by 2 to 3 log values by the milk, but the plaque-forming activities of the adenoviruses and SV40 were not decreased by more than 0.2 log value.

To locate the nonspecific antiviral activities in human milk, SFV was used. This particular virus offered many useful features for our study. (i) Large viral plaques (3 to 4 mm) appeared 40 h after infection on BHK-21 cells, although some small plaques appeared after milk treatment; these may not have been detected with small plaquing viruses; (ii) all plaques were easily detected before and after staining of the cell monolayer with crystal violet; (iii) each infective viral particle produced one plaque (4; we maintained stocks free of detectable defective interfering particles); and (iv) no IgA was expected to be in the milk, which would interfere with our studies (it was reported that antibodies to alphaviruses appeared in only 4/117 adults in our collection area; 1).

Comparison of antiviral activities in different milk specimens. Using SFV as the test virus, we were able to place human milk into two main classes on the basis of heat sensitivity of the antiviral activity of the whole milk (Table 1). The heat-stable component was shown to reside in the cream fraction. Each of the two main classes could be subdivided on the basis of the presence or absence of a heat-labile activ-

ity in the skim fraction (Table 1). Milk of class 1 contained high levels (1.6 to 2.1 $\log_{10}$ reduction of titer) of anti-SFV activity before heating and moderate to high levels (0.7 to 1.9 $\log_{10}$ reduction) after heating. The milk showing a drop in titer after heating did so because of the destruction of the heat-labile skim factor. Class 2 milk had moderate to low (<0.2 to 1.2 $\log_{10}$ reduction) anti-SFV activity before heating and low (<0.2 $\log_{10}$ reduction) activity after heating, due to the absence of any lipid activity and the presence (class 2a) or absence (class 2b) of skim-associated activity. Both the cow and artificial human milk tested were devoid of any kind of activity and, therefore, could be classified as class 2b-type milk.

Overall, we detected antiviral activity in the skim fraction of 50% of the milk samples tested. This activity was destroyed by boiling within 10 min. In 60% of the milk tested, a heat-stable activity located in the cream fraction was detected. Fifteen percent of the human milk samples tested were devoid of any antiviral activity.

Isolation and identification of the heat-stable antiviral activity in the cream fraction. To determine the nature of the heat-stable factor(s), the neutral lipid fraction of cream was extracted. (Negligible activity was found in the phospholipid fraction.) Separation of the neutral lipid fraction by TLC resulted in the detection of seven lipid species (Table 2). Elution and assay of these species indicated that the fatty acid fraction (located by using oleic acid marker) and the monoglyceride fraction (indicated by monolein marker) were the species most active against SFV. No neutral lipid fraction was found to have an effect on Ad-2. The triglyceride fraction (indicated by triolein) was also found to have some antiviral activity, though less than that of the other two fractions, whereas the diglyceride fraction (indicated by 1,3 diolein and 1,2 diolein) had negligible activity. The antiviral activity of oleic acid and monolein was confirmed by directly treating SFV with pure synthetic lipids (Fig. 1). Oleic acid and its glycerol analogs constitute 37% of milk lipids (12). Other fatty acids such as linolein (10% of human milk lipids) and palmitic (27%) were also found to

TABLE 1. *Effect of heat on the antiviral activities of human milk and milk fractions*[a]

Class	No. of individual milk samples	$\log_{10}$ reduction in SFV titer					
		Whole		Cream		Skim	
		Unboiled	Boiled	Unboiled	Boiled	Unboiled	Boiled
1a	6	1.6–2.1	1.6–1.9	1.7–2.1	1.5–2.1	<0.2	<0.2
1b	5	1.7–2.0	0.7–1.2	0.8–1.2	0.7–1.0	0.7–1.0	<0.2
2a	4	0.8–1.2	<0.2	<0.2	<0.2	0.8–1.1	<0.2
2b	3	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Synthetic human milk[b] or cow milk		<0.2	<0.2	<0.2	<0.2	<0.2	<0.2

[a] Assay and separation of cream and skim fractions were performed as described in the text. Whole, cream, or skim milk fractions were boiled in sealed tubes at 100°C for 30 min.

[b] Synthetic human milk (SMA: Wyeth Pharmaceuticals, Parramatta, Australia) was prepared from both powdered and liquid formulations as directed by the manufacturer.

TABLE 2. *Antiviral activity in neutral lipid fractions separated by TLC*[a]

Lipid species	R_f values	% Total of iodine stain[b]	$\log_{10}$ reduction in SFV titer		
			2 mg[c]	10 mg[c]	20 mg[c]
Cholesterol oleate	0.85	<1	0.0	0.0	0.0
Triolein	0.52	20	0.0	0.0	1.0
Oleic acid	0.30	40	1.5	1.9	1.9
Cholesterol	0.15	5	0.0	0.0	0.1
Diolein (1, 3)	0.12	5	0.0	0.0	0.2
Diolein (1, 2)	0.10	20	0.0	0.0	0.2
Monolein	0.0	10	1.9	1.9	1.9

[a] Neutral lipid was extracted and eluted from the plates as described in the text. A 50- to 300-μl volume of lipid solution was streaked onto a silica gel TLC plate, which was developed in n-hexane–methanol–acetic acid (90:10:1 vol/vol) for 2 h.

[b] Unsaturated lipids were visualized after 5 min of iodine vapor treatment and estimated by densitometer tracings.

[c] Total amount of neutral lipid spotted onto TLC plate determined by dry-weight estimations.

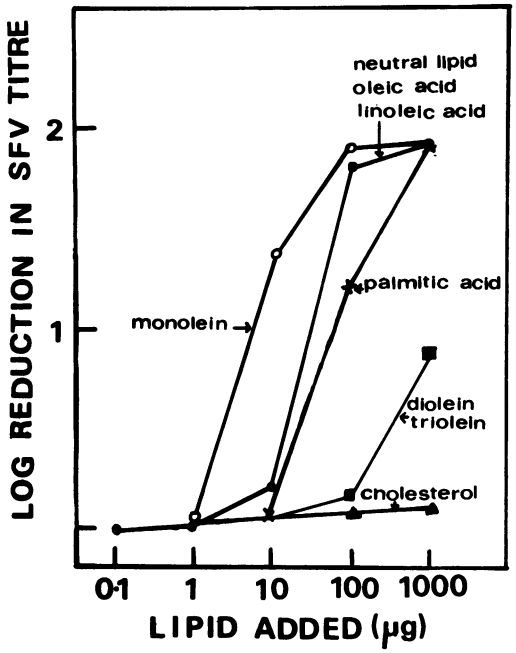

FIG. 1. *Effect of lipids on the activity of SFV. Chloroform solutions of the lipids indicated above (containing 0.1 to 1,000 μg of 99% pure lipid; Sigma Chemical Co.) were dried in sterile tubes. The lipids were resuspended in sterile PBS (0.2 ml), and SFV was added (ca. 200 plaque-forming units in 0.2 ml). After 60 min of incubation at 37°C, 0.2 ml of the total volume was adsorbed to BHK-21 cells for 60 min, and the virus titration was performed as described in the text. The neutral lipid fraction was extracted from the class 1 type milk as described in Table 1. The diolein contained 85% 1,3 diolein and 15% 1,2 diolein.*

have antiviral activity, although palmitic acid was half as active as the unsaturated fatty acids.

When milk lipid extracts from the different classes were separated in a manner similar to that described above using the solvent *n*-hexane–diethyl ether–acetic acid (60:40:2) to clearly separate the monolein from the oleic acid and triolein (and any phospholipids present), it was found that class 1 milk contained nearly three times the concentration of free fatty acids and monoglycerides as did class 2 milk. By contrast, class 2 milk contained almost three times the triglyceride concentration of the class 1 (Table 3). Both cow and artificial human milk again behaved similarly to class 2b milk.

Lipase activity of human milk samples. Since human milk is reportedly capable of strong lipase activity (12), we felt that the difference in glycerol ester composition of milk represented a difference in the lipase activity in the milk samples. We, therefore, examined class 1 and 2 milk, cow milk, and artificial milk for lipase activity as measured by the rate of change of pH resulting from the liberation of butyric acid from tributyrin. Table 3 summarizes the results and demonstrates a tendency for the milk with higher lipase activity (>7 U/ml) to contain higher ratios of monoglycerides and fatty acids:triglycerides than milk having a lower lipase activity (<7 U/ml). The skim milk of class 1a (Table 1) did not have any antiviral activity, but was found to contain 80% of the total milk lipase. This suggests that the lipase alone does not have any anti-SFV activity and its role is to produce lipids that do have antiviral activity (monoglycerides and free fatty acids). Furthermore, the lipase activity was destroyed by boiling for 5 min, and the lipid-associated activity was stable to boiling for 30 min.

TABLE 3. *Relationship between lipid content and lipase activity in human milk*[a]

Milk type[b]	Mean ratio of triglyceride: fatty acid:monoglycerides[c]	Lipase activity[d] (U/ml)
Class 1	20:50:20	7–20
Class 2	70:20:5	<1–7
Synthetic human	80:5:1	<1
Cow	80:5:1	<1

[a] Neutral lipid was extracted and separated by TLC on silica gel plates as described in the text.

[b] Classes of antiviral activity are described in Table 2.

[c] Ratios of tri-, di-, and monoglycerides and other species were estimated by comparing, by densitometer, the densities of the iodine-stained regions on the chromatograms after 5 min of iodine vapor treatment. Total glycerol ester concentration of all milk samples was almost constant (35 to 40 mg/ml) as estimated by glycerol content after lipase digestion using a Roche Centrifican.

[d] Lipase assays were performed as described in the text.

Separation and identification of the heat-labile antiviral activity in the skim fraction of milk. The skim fraction of 50% of the human milk specimens tested contained an anti-SFV activity that was destroyed by boiling for 30 min (Table 1). The skim milk was always devoid of the lipid-associated heat-stable anti-SFV activity. This skim milk-associated antiviral activity was precipitated when the skim milk was made 40% saturated with respect to ammonium sulfate, whereas negligible lipase activity was precipitated by this treatment. Separation of the components of this precipitate on a Sephadex G-200 chromatography column revealed that the antiviral activity was found in the fraction containing the IgA fraction (this fraction being com-

pletely free of lipase activity, Fig. 2). No activity was associated with the IgG or IgM fraction, which are the minority immunoglobulins in human milk (12). Furthermore, this anti-SFV activity was destroyed by treatment with anti-human IgA (α-chain specific) serum or by boiling for 30 min (Fig. 2). Similar results were found in two separate samples.

Characterization of the IgA responsible for anti-SFV activity. It seemed improbable that Melbourne women would have come into contact with SFV (which has yet to be isolated in Australia), but 50% of the samples tested (Table 2) had heat-sensitive anti-SFV activity. From the above results, this activity was thought to be due to cross-reacting IgA antibody. We found that about 5% (1/19 samples tested) of the women's serum tested contained anti-alphavirus neutralizing activity (see also 6; Fig. 3). However, of 30 milk samples tested (Fig. 3), 50% (15/30 samples tested) had neutralizing activity due to antibody. Four women with positive milk samples gave a negative serum response. Samples were considered to have neutralizing activity if they decreased the SFV titer by 66% under the conditions described. This neutralizing activity in the skim milk fraction was decreased by boiling for more than 5 min. When skim milk was tested against various alphaviruses (SFV, Ross River, Getah, Bebaru, and

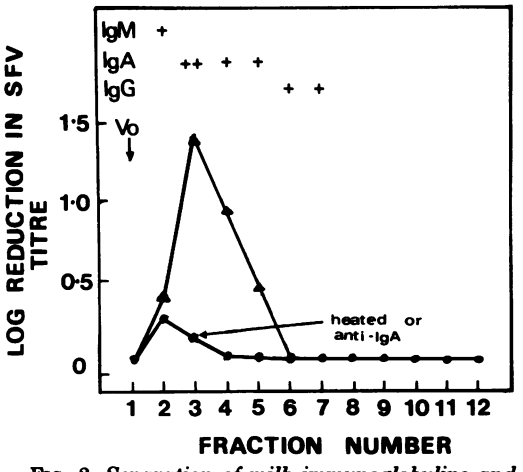

FIG. 2. *Separation of milk immunoglobulins and assay of anti-SFV activity. Ammonium sulfate-precipitated (40% saturation) protein fraction was fractionated by chromatography on Sephadex G-200 as described in the text. The methods of detection of immunoglobulins and assays for antiviral activity are also described in the text. Assays for antiviral activity were performed on untreated samples, samples boiled for 30 min, and samples treated with 200 μg of antihuman (α-chain specific) IgA (Boeringer-Hoescht) for 30 min at 37°C. The anti-IgA serum alone had no effect on SFV titers.*

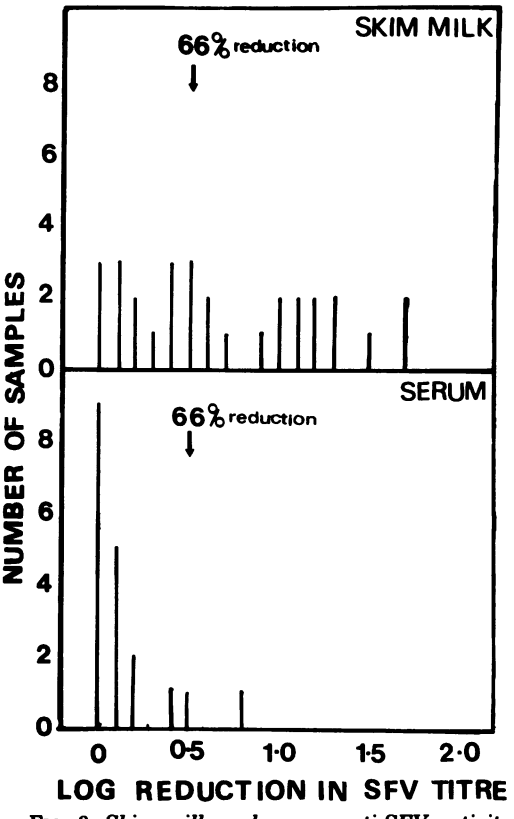

FIG. 3. *Skim milk and serum anti-SFV activity. Skim milk fractions were assayed for antiviral activity as described in the text and were compared with the neutralizing activity of serum samples from comparable women. Serum was incubated at 56°C for 30 min as described by Anderson et al. (1) before assaying as for skim milk. Milk and skim milk samples usually contained 10 to 25 mg/ml of IgA as measured by radial diffusion on S-Partigen IgA immunodiffusion plates (Boeringer-Hoescht; see 14).*

Sindbis), it was found that the Bebaru and Sindbis viruses were not neutralized (Table 4). However, IgA in the skim milk partly neutralized Getah and strongly neutralized Ross River (Table 4), the latter virus being the agent thought responsible for viral polyarthritis (5). The antiviral activity against SFV, Ross River, and Getah was found to have no relation to the lipase activity of the milk sample (Table 4), but paralleled neutralization data reported by Doherty et al. (5) of rabbit antiserum against Ross River.

DISCUSSION

The individual antiviral activities of human milk have yet to be assigned a precise clinical significance, although it is clear that the morbidity in breast-fed infants is less than in those receiving other foods (6, 18). SFV has proved

TABLE 4. *Effect of skim milk on alphavirus titer*[a]

Virus	Log_{10} reduction in virus titer		
	1[b,c]	2	3
SFV	1.0	0.6	0.7
Ross River	1.8	2.1	2.2
Getah	0.5	0.4	0.5
Bebaru	0.2	0.0	0.1
Sindbis	0.3	0.2	0.1

[a] Neutralization assays were performed on BHK-21 cells as described in the text, except that the titer of the untreated virus samples was approximately 10^5 plaque-forming units per ml for all samples used.

[b] Skim milk specimen number. Lipase activity of the samples was 18, 10, and 4 U/ml, respectively.

[c] Cream fraction from this sample decreased all alphavirus titers by 1.7 to 2.1 log_{10} units.

to be a useful tool with which to explore the lipid-mediated antiviral activity of milk. The key to the ability of a given milk to act nonspecifically against viruses appears to lie with the composition of the lipid fraction. Thus, milk comparatively rich in free fatty acids and monoglycerides showed moderate to strong activity, whereas those with a predominance of triglycerides showed low activity (Table 1 and 3). It seems that it is for this reason (i.e., high triglycerides, low monoglycerides) that cow and synthetic milk lack a lipid-mediated activity. Furthermore, there seems to be a correlation between the presence of fatty acids and the degree of lipase activity in milk such as to allow one to assess the likelihood of antiviral activity by a rapid lipase assay. Our findings with the lipid-associated antiviral activity have a particular relevance to the examination of human milk samples for the putative human breast cancer virus (2, 21), as it has previously been reported that human milk will destroy the infectivity of the murine mammary tumor virus (20).

In addition to the lipid-associated activity, we also detected an anti-SFV activity present in the skim fraction that was precipitated in 40% saturated ammonium sulfate. This factor was heat sensitive and eluted with the IgA fraction after Sephadex G-200 chromatography. We confirmed that the antiviral activity was due to IgA by treatment of the IgA fractions with anti-human IgA serum, which destroyed this activity.

Previous (infrequent) reports of alphavirus activity in southern Victoria (e.g. 1) have suggested that only a small proportion of residents (3%) have come in contact with such viruses. This was determined by classical serological techniques. Surprisingly, however, by using IgA from breast milk rather than IgG from blood serum, we found that 50% of women tested possessed SFV neutralizing activity. Moreover, when assayed against a range of alphaviruses, the IgA showed the strongest activity against Ross River virus. A nonspecific effect of IgA appears to be ruled out by the difference in neutralization indexes among related viruses (Table 4). In contrast to our findings, Falkler et al. (9) reported that women with serum antibodies to dengue (flavivirus) did not contain antibodies to this virus in their milk. Thus, it would certainly appear from our results that alphavirus infection in southern Victoria is significantly higher than previously thought. It may, therefore, prove valuable from an epidemiological point of view to examine milk, where available, for IgA in addition to performing the standard blood serum antibody tests.

ACKNOWLEDGMENTS

We thank J. J. Roxon for helpful discussions and D. Leslie of the Royal Women's Hospital for supplying human milk samples.

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