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Effect of Fractions of Ethiopian and Norwegian Colostrum on Rotavirus and *Escherichia coli* Heat-Labile Enterotoxin

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Samples of colostrum from both Ethiopian and Norwegian women contained antirotavirus activities of immunoglobulin and non-immunoglobulin nature. No significant differences in rotavirus immunoglobulin A or in rotavirus-inhibiting activity were found between samples from the two countries. The non-immunoglobulin inhibitory activity was trypsin sensitive and heat stable (100°C for 10 min). *Escherichia coli* heat-labile enterotoxin antibodies were measured in the colostrum samples by enzyme-linked immunosorbent assay. No *E. coli* enterotoxin-specific immunoglobulin A was detected, possibly due to the high background caused by the nonspecific adsorption of immunoglobulin A to the enzyme-linked immunosorbent assay plates in the absence of toxin. A total of 5 of 15 Ethiopian colostrum samples and 0 of 11 Norwegian colostrum samples neutralized the effect of *E. coli* heat-labile enterotoxin on YI adrenal cells. Both the Ethiopian and the Norwegian colostrum samples contained a non-immunoglobulin enterotoxin-inhibitory activity when the toxin was measured by enzyme-linked immunosorbent assay. This inhibitory activity was not trypsin sensitive, and extraction by chloroform-methanol indicated that the inhibitor was of a lipid nature.

It is well documented that human milk protects children from gastrointestinal infections (for references see reference 5), and the milk contains several factors which may be of importance for resistance against infections (6, 14, 31).

Since rotavirus was discovered in 1973 (1), it has been established as one of the major causes of gastroenteritis in children (12). Antibodies against rotavirus have been identified in human milk (2, 22, 23, 26, 35), and recent reports from our laboratory (17) and Totterdell et al. (29) indicate that the milk may also contain rotavirus-inhibiting activity of a non-immunoglobulin nature.

Enterotoxigenic *Escherichia coli* is another cause of serious diarrhea in infants. The bacteria may produce a heat-labile toxin (LT) similar to cholera toxin or a low-molecular-weight heat-stable toxin (ST) or both (20). Lately, such toxin-producing *E. coli* have been associated with diarrhea in children in several parts of the world (7, 10, 18), and LT-producing *E. coli* strains have been isolated from children with diarrhea in Norway (Otnæss and Halvorsen, Acta Pathol. Microbiol. Scand. Sect. B, in press). Antibodies in milk against LT have been reported (9, 24), and recently we have described an *E. coli* LT-inhibitory activity of a non-immunoglobulin nature in mature (later than day 5 postpartum) Norwegian human milk (16).

Thus, there are specific immunoglobulins as well as inhibitory compounds of a non-immunoglobulin nature against both rotavirus and enterotoxigenic *E. coli*. To obtain information about the possible biological roles of these principles, we decided to investigate colostrum samples from two populations presumed to be exposed to these agents to a very different degree. Since we wanted to study the effect of antibodies and other factors separately, some of the colostrum samples were fractionated.

MATERIALS AND METHODS

Colostrum samples. After freezing and thawing, colostrum (day 2 postpartum) from 14 Ethiopian and 11 Norwegian healthy women was centrifuged to remove cells and fat. Five of the Ethiopian and five of the Norwegian colostrum samples were fractionated as described previously (17). Briefly, ammonium sulfate was added to 50% saturation. After centrifugation, the sedimented immunoglobulin-enriched fraction was dissolved in 0.01 M phosphate buffer (pH 7.4) and dialyzed against the same buffer. The non-precipitated fraction was dialyzed against the same buffer. Both fractions were concentrated about 10-fold, and the immunoglobulin-enriched fraction was submitted to column chromatography with Ultrogel AcA44 or

AcA34 equilibrated with phosphate-buffered saline (PBS) (pH 7.4).

The non-precipitated fraction of Ethiopian milk no. 12 was gel filtered through an AcA34 column (65 by 1.6 cm). Each of the two protein peaks eluted was concentrated, and one of them was rechromatographed through a Sephacryl gel S-300 (Pharmacia, Uppsala, Sweden) column (80 by 1.6 cm) at high ionic strength (0.5 M phosphate buffer [pH 7.4] and 1 M NaCl). The protein peak obtained was concentrated and dialyzed against PBS.

To test whether the inhibitory activities of rotavirus and LT were of protein or lipid nature, Norwegian colostrum (day 5 postpartum) was fractionated as described above, and samples of milk fractions (0.39 mg in 0.26 ml) with LT-inhibitory activity or rotavirus-inhibitory activity were incubated with trypsin (Koch-Light Laboratories, England) (7.8 μg in 7.8 μl) for 35 min at 37°C. The reaction was stopped by the addition of soybean trypsin inhibitor (Koch-Light) (78 μg in 7.8 μl). Samples of PBS treated identically served as controls.

Lipid extraction. Milk fractions with rotavirus-inhibitory or LT-inhibitory activity were treated with 19 volumes of chloroform-methanol (2:1) by the method of Folch et al. (4). The organic phase was evaporated with N_2. PBS (260 μl) was added, and the extracted material was suspended by mixing on a Vortex mixer.

Antisera. Swine anti-human immunoglobulin A (IgA) and alkaline phosphatase-conjugated swine anti-human IgA were purchased from Orion Diagnostica, Helsinki, Finland. Anti-lactoferrin was obtained from Dakopatts, Denmark. Swine anti-rabbit IgG (Dakopatts) was coupled to alkaline phosphatase (type VII; Sigma Chemical Co., St. Louis, Mo.) as described previously (30). Burro anti-cholera toxin was kindly provided by John Robbins, Bureau of Biologics, Bethesda, Md.

Rabbit anti-cholera toxin was prepared in our laboratory by subcutaneous injections of 20 μg of purified cholera toxin (Schwarz/Mann, Orangeburg, N.Y.). The first injection was given in Freund complete adjuvant, and the second (after 6 weeks) and the third (after 2 weeks) were given in incomplete adjuvant. Rabbit anti-LT was prepared by two injections of 10 μg of cholera toxin followed by two injections of 400 μg of crude LT in Freund incomplete adjuvant.

IgA against rotavirus. Anti-rotavirus IgA was measured by an indirect immunofluorescence assay against bovine rotavirus-infected BSC-1 cells as previously described (15, 17) and by enzyme-linked immunosorbent assay (ELISA).

ELISA for rotavirus IgA. The Lincoln (Nebraska calf diarrhea virus) strain of calf rotavirus was kindly supplied by P. Halonen, Turku, Finland, and was grown in culture of LLC MK2 cells (21). Virus antigen was prepared from virus-infected cells scraped off of a bottle (200-cm^2 surface) into 8 ml of Hanks balanced salt solution and sonicated three times for 15 s in a Sonifier B-12 (Branson Sonic Power Co., Danbury, Conn.) at 75 W. After low-speed centrifugation, the supernatant was mixed with an equal volume of chloroform. The aqueous phase after centrifugation constituted the antigen. As a result of checkerboard titrations, the antigen was diluted 1:100 for the ELISA.

The ELISA was performed as described by Voller et al. (30). Antigen and control antigen prepared from uninfected LLC MK2 cells were diluted in carbonate buffer (pH 9.6), and 0.1-ml volumes were added to the wells of microtiter plates (Microelisa; Dynatech, Zug, Switzerland). After overnight incubation at 4°C, serial dilutions of milk or milk fractions in PBS-Tween with 2% fetal bovine serum were added, and the plates were incubated for 2 h at 37°C. Alkaline phosphatase-conjugated swine anti-human IgA diluted 1:100 was added, and the plates were incubated for 1 h at 37°C. Before addition of the substrate, the wells were rinsed eight times. The color reaction was stopped by adding 3 M NaOH after 0.5 h, and the plates were read in an eight-channel photometer (Titertek Multiskan; Flow Laboratories, Irvine, Scotland). A rotavirus IgA-positive serum and a negative serum were run with each test. The rotavirus IgA titer of a sample was considered as the highest dilution of the sample where the positive/negative ratio (P/N value) between the absorbance values of the antigen well and the control antigen well was more than 2.

Rotavirus-inhibiting activity. Rotavirus-inhibiting activity was measured against human rotavirus in LLC MK2 cells by the method of Thouless et al. (28) with slight modifications (17).

***E. coli* LT.** *E. coli* LT was measured either by the YI adrenal cell test (3) in a miniculture modification (19), by ELISA (33) as described previously (16), or by GM1 ELISA (25). Round-bottom polyvinyl microtiter plates coated with burro anti-cholera toxin (diluted 1:2,000) or flat-bottom polystyrene microtiter plates coated with ganglioside GM1 (1 μg/ml; Supelco, Bellefonte, Pa.) were used. The GM1-coated plates were incubated overnight at 4°C with 1% bovine serum albumin (Sigma) before use. The coated polyvinyl and polystyrene plates were further treated identically, and the subsequent incubation steps were performed at 37°C for 1 h. Crude LT was diluted in 0.15 M NaCl containing 0.05% bovine serum albumin. Samples of diluted LT (50 μl) were added to the plates together with 50 μl of PBS-Tween, and the plates were incubated and washed before the addition of rabbit anti-LT or rabbit anti-cholera toxin (diluted 1:100). After another incubation, swine anti-rabbit IgG conjugated to alkaline phosphatase was added, followed by substrate after 1 h. The reaction was terminated as described above. The color reaction of the GM1-coated polystyrene plates was read in a Titertek Multiskan, and the results from the antibody-coated polyvinyl plates were recorded in a Beckman spectrophotometer at 405 nm after dilution (1:7) with 0.1 N NaOH.

Measurement of colostrum LT-inhibiting activity. Colostrum LT-inhibiting activity was measured by ELISA essentially as described previously (16). The colostrum samples or fractions were mixed with an equal volume of crude LT and, after 30 min at room temperature, samples (50 μl) were added to antibody-coated or GM1-coated microtiter plates together with PBS-Tween (50 μl). The test was further performed as described above. The colostrum samples were always negative when tested without LT as neg-

ative controls. The inhibition of LT was calculated as the percent decrease of the optical density at 405 nm compared with LT without colostrum fraction. A linear standard curve was obtained with dilutions of crude LT (0.18 g/liter).

LT-inhibiting activity. LT-inhibiting activity was also measured by inhibition of the effect of LT on YI adrenal cells after the addition of a preincubated (30 min) solution of LT and colostrum (16).

Colostrum IgA specific for LT. Colostrum LT-specific IgA was measured by ELISA with either antibody-coated or GM1-coated plates. Dilutions of colostrum samples (50 μl) or fractions were added with an equal volume of PBS-Tween, and the plates were incubated for 1 h at 37°C. Whereas 0.05% Tween was used for the other ELISA described, here we used 0.5% Tween in the washing and dilution buffer to reduce unspecific adsorption of IgA to the plates. Alkaline phosphatase-conjugated swine anti-human IgA (diluted 1:100) was added, and finally the substrate was added as described previously. A P/N value for each colostrum was obtained by dividing the spectrophotometric absorbance of the sample by that of a control without colostrum sample. A P/N value greater than 2 was considered positive (23, 33).

Secretory IgA. Secretory IgA was measured by rocket immunoelectrophoresis with purified secretory IgA from human milk as a standard (17).

Lactoferrin. Lactoferrin was measured by rocket immunoelectrophoresis (17) with purified lactoferrin from human milk as a standard. After ammonium sulfate treatment of four milk samples, the supernatant was concentrated, dialyzed four times with 4 liters, and passed through a column of Ultrogel AcA34 (2.6 by 40 cm) and rechromatographed through Ultrogel AcA44 (1.6 by 70 cm). PBS (pH 7.4) was used as the dialysis and elution buffer. The purified lactoferrin preparation was 98% pure as judged by stained gels after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE. SDS-PAGE was performed by the method of Laemmli (11) in 10% polyacrylamide flat or round gels.

Protein. Protein was measured by the method of Lowry et al. (13) with bovine serum albumin (Sigma) as a standard.

Protein solutions. Protein solutions were concentrated with Amicon ultrafiltration equipment with PM10 or XM100 filters.

RESULTS

IgA against rotavirus. With the ELISA technique, rotavirus IgA was detected in 12 of 14 Ethiopian and in all but 1 of 11 Norwegian colostrum samples (Table 1). There was no significant difference in antibody titer: geometric mean titer was 18.4 from the Ethiopian samples as compared with 16 from the Norwegian samples. When the same samples were tested by the immunofluorescence technique, rotavirus IgA could only be detected at very low titers in 11 samples. No rotavirus-specific IgA was detected by either method in the supernatants after ammonium sulfate treatment, whereas in the precipitated fraction rotavirus antibodies were detected by ELISA in all of the Norwegian samples and in four of the five Ethiopian samples (Table 2).

TABLE 1. *IgA and inhibitory activity against rotavirus in Ethiopian and Norwegian colostrum*

Colostrum no.	Total protein (g/liter)	Total IgA (g/liter)	Rotavirus IgA		Rotavirus inhibition[a]
			IFA[b]	ELISA[c]	
Ethiopian					
1	18.2	6	1	16	20
2	80.0	40	2	32	≥40
3	62.0	38	1	16	10
4	17.8	5	0	16	10
5	15.4	6	2	32	20
6	22.8	14	<8[d]	32	40
7	20.4	6	1	8	20
8	27.6	14	<4[d]	32	10
9	28.8	5	2	128	≥40
10	17.8	2	0	<8	<5
11	16.8	5	0	8	<5
12	19.8	8	1	<8	40
13	23.0	40	NT	32	NT
14	13.6	50	2	128	10
Norwegian					
1	16.4	4	2	16	40
2	16.0	3	0	8	20
3	34.8	19	1	64	160
4	17.6	6	0	8	20
5	10.4	8	0	16	20
6	37.8	11	0	32	10
7	24.4	10	0	<8	10
8	52.5	20	4	128	160
9	26.8	12	NT	16	20
10	NT	NT	1	16	<5
11	NT	NT	0	8	20

[a] Inhibition is expressed as the highest dilution which reduced the number of infected cells by 50%.

[b] IFA, Rotavirus IgA expressed as the highest dilution with positive immunofluorescence staining; 0, negative when sample tested was undiluted; NT, not tested.

[c] ELISA, Rotavirus IgA expressed as the highest dilution with a difference in P/N value of 2 between the antigen well and the control well.

[d] Testing at lower dilutions was not possible in the immunofluorescence assay due to unspecific staining.

Gel filtration of the ammonium sulfate-precipitated fraction resulted in three protein peaks (Fig. 1); IgA was eluted in peak I, and peak III was devoid of any IgA (17) (Table 3). As compared with mature milk, peak III was considerably smaller in colostrum (data not shown). IgA against rotavirus was detected in all the Ethiopian peak Ib fractions and in four out of five Norwegian peak Ib fractions (Table 3).

Rotavirus inhibition. Rotavirus-inhibitory activity was detected in 11 of the 13 Ethiopian colostrum samples tested and in 10 of the 11 Norwegian samples (Table 1). Antivirus activity

TABLE 2. *IgA and inhibitory activity against rotavirus in colostrum fractions*

Colostrum no.	Ammonium sulfate-precipitated fraction					Ammonium sulfate supernatant fraction				
	Total protein (g/liter)	Total IgA (g/liter)	Rotavirus IgA		Rotavirus inhibition[a]	Total protein (g/liter)	Total IgA (g/liter)	Rotavirus IgA		Rotavirus inhibition
			IFA[b]	ELISA[c]				IFA	ELISA	
Ethiopian										
2	27.5	25	≤4[d]	64	160	14.0	0	0	<2	10
5	22.0	16	1	8	20	24.6	0.4	0	<2	10
6	24.0	15	4	128	40	16.4	0.2	0	<4[d]	5
7	15.0	10	0	<2	<5	7.0	0	0	<2	<5
12	12.7	11	<2[d]	4	20	5.8	0	0	<2	20
Norwegian										
2	9.8	6	0	8	10	18.4	3.7	0	<2	5
3	32.0	24	2	64	80	6.8	0.4	0	<10[d]	40
7	25.0	19	0	4	5	6.4	1.0	0	<2	5
8	50.0	20	4	128	80	14.8	0	0	<2	<5
9	19.0	10	2	32	80	8.0	0	0	<2	<5

[a] See footnote *a* of Table 1.
[b] See footnote *b* of Table 1.
[c] See footnote *c* of Table 1.
[d] See footnote *d* of Table 1.

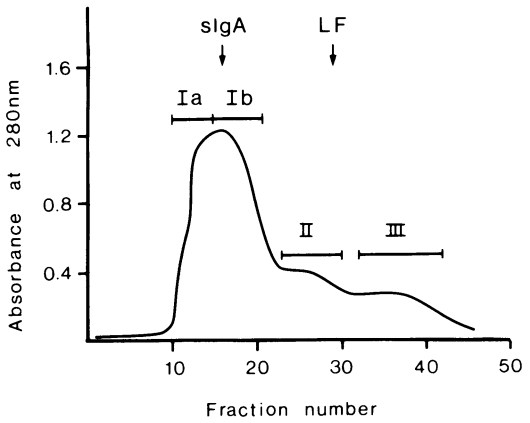

FIG. 1. *Gel filtration of the ammonium sulfate-precipitated fraction from Norwegian colostrum sample no. 4 through an AcA44 column equilibrated with PBS (pH 7.4). Fractions were pooled as indicated.*

was seen in the sediment as well as in the supernatant fractions after ammonium sulfate treatment (Table 2), even though rotavirus-specific IgA was not detected in the non-precipitated fractions. In peak Ib (IgA peak), after gel filtration of the immunoglobulin-enriched precipitated fraction, all but Norwegian milk no. 8 contained inhibitory activity (Table 3). Inhibitory activity was detected in peak III (devoid of IgA) of Ethiopian milk no. 6.

Ethiopian milk no. 12, with relatively high virus-inhibitory activity in the supernatant after ammonium sulfate treatment, was without detectable IgA (Table 2), and a sample was submitted to gel filtration (Fig. 2). Inhibitory activity was present in both protein peaks (Fig. 3) although the major part was eluted in peak b, where 80% of the total protein was lactoferrin. Storage at −20°C and freezing and thawing reduced the activity (data not shown). SDS-PAGE of peak b revealed, besides the major protein band of lactoferrin (60,000 to 70,000 daltons), several minor protein bands, the two most pronounced at 25,000 to 30,000 daltons. A sample (0.3 ml) of peak b (0.54 g/liter) was passed through a Sephacryl S-300 column at high ionic strength (1 M NaCl). One protein peak containing lactoferrin was detected, and after dialysis and concentration (0.6 g/liter), no antirotavirus activity was found, and the two protein bands at 25,000 to 30,000 daltons were no longer observed in the SDS-PAGE.

To further elucidate the nature of the rotavirus-inhibitory activity, samples of Norwegian milk (day 4 postpartum) with rotavirus-inhibitory activity, which was depleted of IgA by ammonium sulfate treatment and gel filtration, were treated with (i) trypsin, (ii) chloroform-methanol, or (iii) heat (10 min at 60, 80, or 100°C). Trypsin destroyed the inhibitory activity, whereas no activity was observed in the chloroform-methanol extract. There was no loss of inhibitory activity after the heat treatment.

IgA specific for *E. coli* LT. Apparently 6 of 13 Ethiopian colostrum samples and 6 of 11 Norwegian samples in 10-fold dilutions contained LT-specific IgA as measured by ELISA when the ELISA plates were coated with GM1 (Fig. 4) or anti-cholera toxin (data not shown). However, when LT was omitted in the test, almost equal amounts of IgA were adsorbed to

TABLE 3. *Rotavirus IgA and inhibitory activity in peak Ib and III after gel filtration through an Ultrogel A44 column in PBS of ammonium sulfate precipitate fractions*

Colostrum no.	Peak Ib					Peak III				
	Total protein (g/liter)	Total IgA (g/liter)	Rotavirus IgA		Rotavirus inhibition[a]	Total protein (g/liter)	Total IgA (g/liter)	Rotavirus IgA		Rotavirus inhibition
			IFA[b]	ELISA[c]				IFA	ELISA	
Ethiopian										
2	4.6	4	1	2	10	1.2	0	0	<2	<5
5	2.5	2	1	16	10	1.7	0	0	<2	<5
6	3.0	3	2	32	10	0.5	0	0	<2	5
7	3.1	3	1	<4[d]	5	0.9	0	0	<2	<5
12	7.1	7	0	<4[d]	10	1.1	0	0	<2	<5
Norwegian										
2	1.0	1	1	2	5	0.2	0	0	<16[d]	<5
3	7.0	7	<4[d]	<8[d]	20	0.6	0	0	<8[d]	<5
7	5.0	5	0	8	10	3.7	0	0	<2	<5
8	2.0	2	NT	8	<5	NT	0	NT	NT	NT
9	1.6	1	0	2	5	0.2	0	0	<2	<5

[a] See footnote *a* of Table 1.
[b] See footnote *b* of Table 1.
[c] See footnote *c* of Table 1.
[d] See footnote *d* of Table 1.

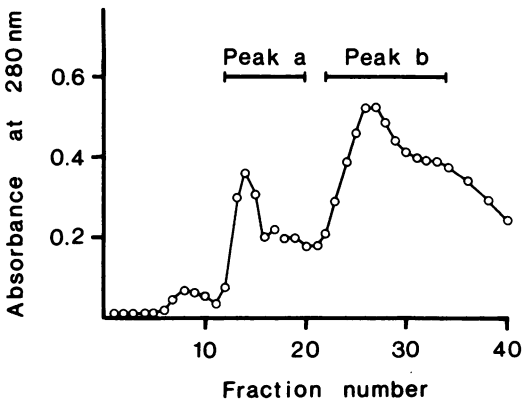

FIG. 2. *Gel filtration of ammonium sulfate supernatant fraction from Ethiopian colostrum sample no. 12 through an AcA34 column equilibrated with PBS (pH 7.4). Fractions were pooled as indicated.*

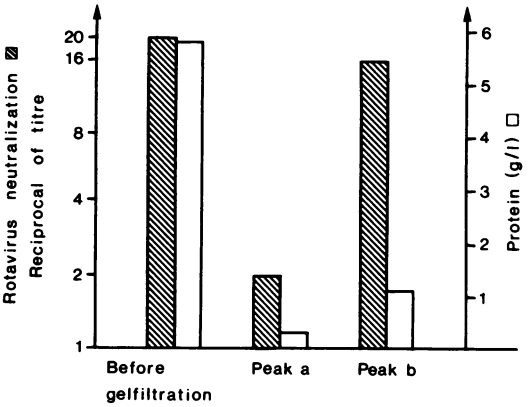

FIG. 3. *Rotavirus-inhibitory activity before and after gel filtration of the ammonium sulfate supernatant fraction of Ethiopian colostrum no. 12. Panels: a, pooled fractions of protein peak of >150,000 daltons; b, pooled fractions of protein peak of >50,000 daltons.*

the wells. In no sample was the difference in P/N value between the LT well and the control well more than 2 (Fig. 4). None of the Norwegian colostrum samples inhibited the effect of LT on YI adrenal cells, whereas undiluted aliquots of the five Ethiopian samples inhibited LT on YI adrenal cells.

LT-inhibiting activity. All of the Norwegian and Ethiopian colostrum samples contained inhibitory activity of LT when the toxin was measured by ELISA. Similar results were obtained whether the ELISA plate was coated with high-titered burro anti-cholera toxin or GM1 (data not shown). This inhibitory activity was only partly precipitated by ammonium sulfate, regularly leaving the major part in the supernatant where the IgA concentration was very low (Table 4), in agreement with our earlier observations with mature Norwegian milk (16).

The toxin-inhibitory activity was eluted from a gel filtration column corresponding to an apparent molecular weight of more than 400,000 (16). This high-molecular-weight activity peak from pooled Norwegian milk samples (4 days postpartum) was exposed to (i) trypsin treatment or (ii) lipid extraction by chloroform-methanol. The inhibitory activity was extracted with the organic phase, whereas trypsin treatment

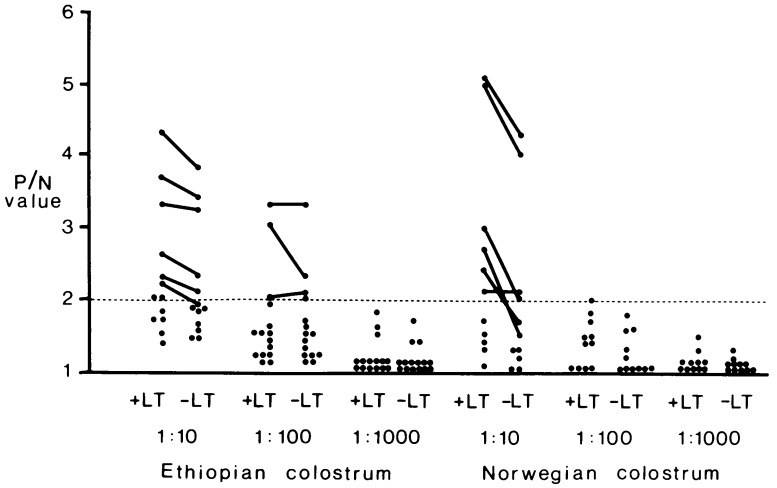

FIG. 4. *LT-specific IgA of colostrum samples measured by ELISA in the presence or absence of LT on the plates. The P/N value is the absorbance at 405 nm of the sample well divided by the adsorbance of a control well without colostrum sample.*

TABLE 4. *Inhibitory effect of colostrum fractions on the binding of LT to ELISA plates*

Colostrum no.	Ammonium sulfate-precipitated fraction		Ammonium sulfate non-precipitated fraction	
	Inhibition of LT (%)[a]	IgA (% of total protein)	Inhibition of LT (%)	IgA (% of total protein)
Ethiopian				
2	80	91	80	0
5	70	85	80	2
6	80	63	100	1
7	80	64	100	0
12	90	87	100	0
Norwegian				
2	70	41	80	5
3	50	76	70	6
7	70	77	70	16
8	80	40	70	0
9	50	52	70	0

[a] Inhibition of LT binding after preincubation of LT with 0.1 g of protein per liter of colostrum fraction.

had hardly any effect (0 to 10%). No protein was measured in the organic extract, and SDS-PAGE revealed no protein band.

DISCUSSION

Ethiopian women may, because of their living conditions, be more frequently exposed to rotavirus infections and reinfections than Norwegian women, and an increased exposure could give rise to higher antibody titers in their colostrum. However, no difference in rotavirus IgA antibody titer could be detected in the present study.

A comparison of the results of the ELISA and the indirect immunofluorescence technique showed that the former was considerably more sensitive than the latter. In previous studies of human milk, investigators using immunofluorescence (17, 22, 26) have detected rotavirus IgA less frequently than those who used radioimmunoassay or ELISA (2, 35).

The reading of endpoint titers by indirect immunofluorescence may be difficult to standardize and may explain the discrepancy observed between the titers obtained by the immunofluorescence and the ELISA method in some samples.

As observed in a previous study (17), we found a correlation between rotavirus IgA and inhibitory activity against human rotavirus. However, the correlation was not complete, and the results from some samples suggested that rotavirus IgA may be present which does not inhibit the human rotavirus (Ethiopian sample no. 11 and Norwegian sample no. 10, Table 1), whereas fractions without detectable rotavirus IgA contained rotavirus-inhibitory capacity (fraction of Ethiopain samples no. 2, 5, and 12, and of Norwegian samples no. 2, 3, and 7, Table 2; peak III of Ethiopian sample no. 3, Table 3). The first observation may be due to variations among the human rotaviruses (27), and the second observation confirms our previous report that the milk may contain antirotavirus activity of a non-immunoglobulin nature (17). In view of a recent report on breast milk antibodies in neonatal rotavirus infection, where it was concluded that factors other than the rotavirus antibodies most probably are of importance in preventing infec-

tion (29), it is of interest to look further into the nature of the non-immunoglobulin rotavirus-inhibitory activity. Antirotavirus activity was observed in the supernatant after ammonium sulfate treatment, where the IgA concentration was very low (Table 2). The inhibitory activity was eluted from the gel filtration column together with lactoferrin (Fig. 2 and 3). The loss of the activity from the lactoferrin peak after gel filtration in 1 M NaCl was accompanied by the disappearance of low-molecular-weight proteins (25,000 to 30,000) as observed in SDS-PAGE, indicating that low-molecular-weight structures rather than lactoferrin were responsible for the inhibitory activity. Lactoferrin is well known to aggregate with other proteins (8), and possibly an inhibitor was noncovalently linked to lactoferrin. Inactivation of the rotavirus-inhibitory activity by trypsin indicated that the inhibitory activity was of a protein nature.

Gel filtration of the ammonium sulfate precipitate material of Ethiopian milk no. 6 revealed a rotavirus-inhibitory activity in peak III (Table 3), with an apparent molecular weight less than 40,000, in agreement with our earlier studies of mature Norwegian milk. At this point we do not know whether this activity is trypsin sensitive.

Since the fat of the colostrum samples was removed before testing and no inhibitory activity was extracted with chloroform and methanol, the antirotavirus neutralizing activity described was most likely different from the lipid-mediated antiviral activity described by Welsh et al. (32).

Five of the Ethiopian colostrum samples and none of the Norwegian samples inhibited LT in a YI adrenal cell assay. Similar results were obtained in a comparison of Pakistani and Swedish milk samples in which 9 out of 15 samples from Pakistani women and none of the Swedish milk samples contained LT-inhibitory activity as measured by the YI adrenal cell assay (9). Unspecific adsorption of colostrum IgA to the wells of ELISA plates (Fig. 2) may have masked low concentrations of LT-specific IgA. Rheumatoid factor has previously been associated with nonspecific reactions in ELISA (34). If rheumatoid factor-IgA complexes were present in the milk, such complexes could bind to antibody-coated plates and in that way cause nonspecific adsorption of IgA to the plates. The fact that similar nonspecific IgA adsorption was observed with GM1-coated plates indicates that rheumatoid factor was not responsible for this effect.

A nonspecific adsorption of IgA was also observed in ELISA for rotavirus and may have influenced the sensitivity of the test. The detection of rotavirus-specific IgA in most samples probably reflects the presence of higher concentrations of rotavirus-specific IgA.

The colostrum samples of Ethiopian as well as Norwegian women contained the non-immunoglobulin-inhibitory activity of *E. coli* LT (measured by ELISA) as we observed in the mature milk (16). The inhibitory activity appeared to be somewhat higher in the Ethiopian samples, either due to individual variations or to the dissimilar living conditions. Great variations were seen in 50 Norwegian milk samples we have analyzed, although all the samples contained some inhibitory activity.

In the high-molecular-weight peak with inhibitory activity obtained after gel filtration, no immunoglobulins were detected in some of the fractionated samples. Yet such milk fractions inhibited LT in vivo in rabbit ileal loops, and a correlation between the inhibitory effect in vitro and in vivo was observed (A.-B. Otnæss and A.-M. Svennerholm, submitted for publication).

Upon extraction of the inhibitory activity by chloroform and methanol, the activity was found in the organic phase, indicating a lipid nature of the LT inhibitor. Elution of the LT inhibitor in the void volume together with protein indicates a protein-lipid complex, whereas the absence of protein and the presence of inhibitory activity after chloroform-methanol extraction suggest that protein is not of vital importance for the activity. The lack of effect of trypsin treatment is in agreement with the activity being of a nonprotein nature.

The non-immunoglobulin factor(s) responsible for the rotavirus-inhibiting activities appeared to be associated with protein fractions of the milk, whereas the LT-inhibiting activity was associated with lipid structures. Whether it is known components of the milk that exert the observed inhibitory activities has to be explored further. The fact that all the Norwegian and Ethiopian milk samples show LT-inhibitory activity indicates that this activity may be associated with a common constituent of human milk.

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