Inhibitory Effects of Human and Bovine Milk Constituents on Rotavirus Infections

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ABSTRACT

Among etiologic agents, rotavirus is the major cause of severe dehydration diarrhea in infant mammals. In vitro and in vivo studies have indicated that the human milk-fat globule protein lactadherin inhibits rotavirus binding and protects breast-fed children against symptomatic rotavirus infection. The present work was conducted to evaluate the effect of lactadherin, along with some other milk proteins and fractions, on rotavirus infections in MA104 and Caco-2 cell lines. It is shown that human, and not bovine, lactadherin inhibits Wa rotavirus infection in vitro. Human lactadherin seems to act through a mechanism involving protein-virus interactions. The reason for the activity of human lactadherin is not clear, but it might lie within differences in the protein structure or the attached oligosaccharides. Likewise, in our hands, bovine lactoferrin did not show any suppressive activity against rotavirus. In contrast, MUC1 from bovine milk inhibits the neuraminidase-sensitive rotavirus RRV strain efficiently, whereas it has no effect on the neuraminidase-resistant Wa strain. Finally, a bovine macromolecular whey protein fraction turned out to have an efficient and versatile inhibitory activity against rotavirus.

(**Key words:** rotavirus, milk fat globule membrane protein, lactoferrin, whey protein)

Abbreviation key: DMEM = Dulbecco's minimal essential medium with Glutamax, EGF = epidermal growth factor, MFGM = milk-fat globule membrane, MMWP = macromolecular whey proteins, PAS = periodic acid Schiff reagent.

INTRODUCTION

Rotavirus is a global pathogen infecting mammals. Worldwide, 95% of all children are infected before the

age of 5, resulting in about half a million deaths annually. Improved hygienic and sanitary conditions do not affect the incidence of rotavirus infections. The impact is very high in developing countries, whereas better medical care reduces lethality in developed countries. Still, the virus represents a serious health problem, leading to substantial economic expenses when the negative effect on livestock production is also taken into account. Efforts to develop vaccines against rotavirus have been under way since 1980. To date, no efficient treatment is available (for reviews see Jiang et al., 2002; Kosek et al., 2003; Parashar et al., 2003). Rotavirus infection occurs in the small intestine and is restricted to the upper and mid part of the villi in mature enterocytes. Knowledge continues to accumulate about the complicated process of rotavirus binding and infection of the cell (for review see Arias et al., 2002).

During the past decade, there has been a lot of focus on bioactive components in milk. Investigations using cell cultures have shown the ability of human milk fractions to inhibit rotavirus replication. Considerable effects were reached by a mucin complex fraction containing the milk-fat globule membrane (MFGM) proteins MUC1, butyrophilin, and lactadherin. Based on experiments with virus-binding abilities, it was suggested that lactadherin might be responsible for the action of the mucin complex (Yolken et al., 1992). However, the work by Yolken and coworkers did not provide direct proof for the action of lactadherin.

The in vitro studies were followed by a cohort study to evaluate the correlation between lactadherin in breast milk and symptomatic rotavirus infection (Newburg et al., 1998). A logistic regression model, adjusted for secretory IgA and age, disclosed a significant difference in the lactadherin concentration of milk received by symptomatic (29.2 µg/mL) and asymptomatic (48.4 µg/mL) infected babies.

Another research group performed in vitro experiments examining the existence of rotavirus inhibitory components in bovine milk (Kanamaru et al., 1999). A high molecular weight fraction from bovine whey protein demonstrated antiviral effect. This fraction con-

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tained MUC1, lactadherin, and an unidentified 80-kDa protein. The same study examined effects of a human milk fraction prepared in a similar manner, except that an affinity column was included to remove IgG. This fraction, containing mostly MUC1, possessed antiviral activity when assayed for effects on replication of 3 human rotavirus strains (Kanamaru et al., 1999).

Lactadherin exists in bovine MFGM in 2 glycosylation forms: A 52-kDa variant glycosylated at Ser9, Asn41, and Asn209; and a 47-kDa variant with glycans at Thr16 and Asn41 (Hvarregaard et al., 1996). This and orthologous proteins have been named PAS-6/7, component 15/component 16, MGP57/MGP53, bovine-associated mucoprotein, BA-46, P47, and MFG-E8 (Mather, 2000). Lactadherin comprises 2 N-terminal epidermal growth factor (EGF) homology domains, and 2 repeated C domains sharing homology with the discoidin family including the lipid-binding C1 and C2 domains of blood coagulation factor VIII and factor V (Stubbs et al., 1990). The second EGF domain contains an Arg-Gly-Asp motif demonstrated to interact with the $\alpha v\beta 5$ and $\alpha v\beta 3$ integrins, whereas the second C domain binds to anionic phospholipids (Andersen et al., 1997). Between species, lactadherin proves to be structurally well conserved, except for human lactadherin, which lacks the first EGF-domain.

As described above, there has been a considerable amount of indirect evidence published for the action of lactadherin and other milk proteins on rotavirus infection. The aim of the present study was to perform *in vitro* experiments with pure milk proteins and to investigate effects of some interesting milk fractions on rotavirus infection.

MATERIALS AND METHODS

Materials

All chemicals (analytical grade) were supplied by Sigma-Aldrich Corp. (St. Louis, MO) or Merck & Co., Inc. (Whitehouse Station, NJ), unless otherwise specified. All tissue culture media and additives were purchased from Gibco Invitrogen Corp. (Paisley, UK), unless otherwise specified. Culture plates were from BD Falcon (BD Biosciences Discovery Labware, Boston, MA). Pure bovine lactoferrin was supplied by DMV International Nutritionals (Veghel, The Netherlands).

Protein Purification

All procedures were carried out at 4°C, unless otherwise indicated. Human lactadherin was purified by modification of a previously published method (Taylor et al., 1997). Briefly, cream was obtained by centrifugation of human milk for 20 min at 5000 $\times g$. The separated

cream was subsequently washed with 10 mM Na₂PO₄, pH 7.2, 0.15 M NaCl (PBS), and after another centrifugation (5000 $\times g$ for 20 min), was resuspended in 5 volumes of PBS. Vigorous churning and sonication were used to release MFGM, which was subsequently pelleted by ultracentrifugation at 100,000 $\times g$ for 1 h. Proteins were solubilized by resuspending the isolated membranes in PBS (4 mg of protein/mL) with 2.5% Triton X-114 (vol/vol), sonicating, and mixing overnight. Phase separation was obtained by heating at 37°C for 1 h followed by centrifugation (20 min, 4000 $\times g$, at 25°C). The lower detergent phase was collected and the protein was precipitated with 10 volumes of acetone overnight at -20°C, followed by centrifugation (10,000 $\times g$, 15 min). Additional purification was achieved by reverse phase chromatography using a 1-mL Resource RPC column (Amersham Biosciences, Uppsala, Sweden) and application of a linear gradient from 0 to 80% 2-propanol in 20% formic acid. Before injection, the material was dissolved in 20% formic acid. The lactadherin-containing fractions were diluted 4 times with water and then freeze-dried.

Bovine lactadherin and MUC1 were purified according to Hvarregaard et al. (1996) and Pallesen et al. (2001), respectively.

A fraction of bovine macromolecular whey proteins (MMWP) equals the retentate (Lacprodan MFGM-10) obtained by microfiltration (0.1 to 0.2 μm) of whey from commercial yellow cheese production. This and other whey protein fractions were produced and kindly donated by Arla Foods Ingredients, Nr. Vium, Denmark.

Proteins were visualized by SDS-PAGE (18%) using standard procedures and stained with Coomassie Brilliant Blue R-250, periodic acid Schiff reagent (PAS), or silver. Identity and purity was documented by N-terminal amino acid sequencing on a Procise Protein Sequencer (Applied Biosystems, Foster City, CA); quantification was done by amino acid analysis (OPA-based) or by a modified Lowry method (Schacterle and Pollack, 1973).

Rotavirus Propagation

The embryonic monkey kidney cell line MA104 (DSMZ, Braunschweig, Germany) was used to propagate rotavirus stock solutions of the Wa (human) and RRV (simian) strains. MA104 cells were cultured in Dulbecco's minimal essential medium with Glutamax (DMEM), supplemented with 10% fetal calf serum, and 1% antibiotics (equivalent to 100 U of penicillin/mL of medium and 100 mg of streptomycin/mL of medium), hereinafter referred to as supplemented DMEM. Cells were grown in cell culture plates at 37°C in a 5% CO₂ incubator. Confluent cell monolayers were infected (1

h and shaken every 15 min) after washing twice with DMEM with preactivated virus (10 $\mu\text{g/mL}$ of TPCK-treated trypsin for 30 min). The viral inoculum was subsequently removed and the cells were incubated 1 to 2 d until complete cytopathic effect was achieved. The virus/cell solution was subsequently centrifuged ($3000 \times g$ for 5 min) to remove cells and cell debris residues; aliquots of virus supernatant were stored at -80°C .

Rotavirus Infection Assays

The in vitro infection assays using MA104 or Caco-2 cells (DSMZ, Braunschweig, Germany) were performed essentially as previously described (Jourdan et al., 1995; Pando et al., 2002). Cells were grown to approximately 80% confluence in 96-well plates in supplemented DMEM at 37°C in an incubator with 5% CO_2 . The plates were then washed twice with DMEM and incubated for 24 h in DMEM. Inoculum (80 $\mu\text{L/well}$) consisting of rotavirus (giving ~ 200 primary infections/well) with or without (control) designated amounts of milk protein samples (all in DMEM) was administered and left on the cells for 1 h at 37°C . After removal of the inoculum, the cells were washed once with DMEM to remove unbound virus before production of virus progeny for 18 to 22 h at 37°C in 5% CO_2 (standard infection conditions). Cells infected with rotavirus (focus-forming units) were identified by immunoperoxidase/Carbazol-staining as described by Arias et al. (1987), and evaluated microscopically.

Rotavirus Inhibition Mechanism

To determine at which step an antiviral component interferes with the infectious process of rotavirus, binding and infection assays were performed as described by Zarate et al. (2000). The Caco-2 cells were cultured as described, but the washing steps and dilutions were performed with cold DMEM instead of warmed (to 37°C) medium. Three types of binding assays were performed. Firstly, in a protein/cell preincubation assay, serial dilutions of milk proteins or fractions were allowed to interact with the cell surface by incubating the culture plate with protein at 4°C for 1 h. After removal of the protein solutions, the cells were washed once and incubated with activated virus for 1 h at 4°C , and virus infection was monitored at standard conditions. The second type of assay was done by preincubating virus and protein solutions for 1 h at 4°C , before transferring them to cold and washed cells for an additional hour of incubation at 4°C and allowing virus infection under standard conditions. Third, a postattachment assay was performed by preincubating virus with cells (1 h

at 4°C) before performing a standard virus infection assay. Controls were performed by replacing protein solutions with DMEM. Subsequent identification of infected cells was done as described in the previous section.

Quantification of Bovine Lactadherin and MUC1

Contents of bovine lactadherin in milk and milk fractions were measured by a sandwich enzyme-linked immunosorbent assay. Microwell plates (96F, Nunc A/S, Roskilde, Denmark) were coated overnight at 4°C with 4 $\mu\text{g/mL}$ (100 $\mu\text{L/well}$) of rabbit antilactadherin antibodies (Andersen et al., 1997) in 0.1 M Na_2CO_3 , pH 9.8. After washing 3 times with PBS and blocking using the same buffer with 0.5% gelatin (wt/vol) at 37°C for 2 h, the plates were washed 3 times with PBS containing 0.1% Tween 20 (vol/vol). Samples and standards (100 $\mu\text{L/well}$) were then administered diluted in PBS with 0.1% Triton X-100 (vol/vol) and incubated at room temperature for 2 h. With intermediate 3 times washing with PBS-Tween, the following steps involved sequential addition of 1) biotinylated rabbit anti-bovine lactadherin IgG in PBS-Tween (100 $\mu\text{L/well}$, 0.5 $\mu\text{g/mL}$, and 37°C for 1 h), 2) horseradish peroxidase-conjugated streptavidin (P0397, DakoCytomation Norden A/S, Glostrup, Denmark) diluted 1:2000 in PBS-Tween (100 $\mu\text{L/well}$, 37°C for 1 h), and 3) sustaining with 100 $\mu\text{L/well}$ of the chromophor ortho-phenylenediamine (0.5 mg/mL) in 0.15 M Na-citrate, pH 5.0. The reaction was stopped with 100 μL of 1 M H_2SO_4 , and the absorbance at 490 nm was measured. Linearity was obtained when standards (lactadherin) were used in a range from 0.4 to 12.5 ng/mL.

Amounts of bovine MUC1 were estimated by densitometric scanning of Western blots, performed as described before (Benfeldt et al., 1995), and subsequently analyzed using a flat bed scanner and the QuantiScan 2.1 software (Biosoft, Cambridge, UK). Standards (MUC1) were used in a range from 0.2 to 4.0 ng/lane. Pure bovine MUC1 was used to raise polyclonal antibodies (DakoCytomation Norden A/S).

Statistical Analysis

Effects of tested proteins or protein mixtures on rotavirus infectivity in relation to controls (no protein) were tested for statistical significance by using Student's *t*-test. Significance was defined as $P < 0.05$.

RESULTS

Protein Purification

Human lactadherin was purified from human MFGM by Triton X-114 extraction followed by reverse phase

Figure 1. Protein purification. Human and bovine milk proteins were purified as described. (A) The final reverse phase HPLC step in purification of human lactadherin was performed on a 1-mL Resource RPC column and the protein eluted with a gradient of 0 to 80% 2-propanol in 20% formic acid at 40°C (dotted line). Proteins were monitored at 280 nm (solid line). The peak containing human lactadherin is indicated (arrow). (B) Sodium dodecyl sulfate-PAGE gels showing the source material and obtained protein fractions. Gels were stained with Coomassie Brilliant Blue R-250 (Co), periodic acid Schiff reagent (PAS), or silver (Ag). Lane 1, human milk fat globule membrane (MFGM) (Co); lane 2, human MFGM (PAS); lane 3, human lactadherin (Co); lane 4, human lactadherin (Ag); lane 5, bovine MFGM (Co); lane 6, bovine MUC1 (PAS); lane 7, bovine lactadherin (Co); lane 8, MMWP (Co); and lane 9, MMWP (PAS).

chromatography. Remarkably, human lactadherin behaves as a transmembrane protein because it is found in the detergent phase. Although the human lactadherin appeared to be relatively pure, an additional purification step using reverse phase chromatography was introduced (Figure 1A), which assured a very high purity of the protein (Figure 1B, lane 3). Only a few other faint bands can be seen when the purity of the human lactadherin was tested by overloading and silver stain-

Figure 2. Dose-response of different lactadherin-containing milk fractions on RRV rotavirus infectivity of MA104 cells in relation to controls (no protein). Human milk fat globule membrane (MFGM); bovine MFGM; and bovine macromolecular whey proteins (MMWP). Results are shown as mean $\pm$ standard deviation of triplicates. First point at which a statistically significant ($P < 0.05$) effect was obtained is indicated with an asterisk (*).

ing an SDS-PAGE gel (Figure 1B, lane 4). Density scanning of Coomassie- or silver-stained gels showed that lactadherin amounts to 94 to 98% of the present protein preparation. Furthermore, analysis of the purified sample by N-terminal amino acid sequencing confirmed the identity of the isolated protein as human lactadherin (EMBL accession number Q08431) and showed no trace of any contaminant (i.e., all below 5%). Interestingly, human lactadherin migrates as 2 bands like the bovine protein (Figure 1B, lane 7). In addition, pure samples of bovine lactadherin and MUC1 were generated (Figure 1B, lanes 6 and 7), to test the effects of these proteins on rotavirus infectivity.

Bovine Lactadherin Does Not Inhibit RRV Infection of MA104 Cells

We tested the effects of lactadherin-containing milk protein fractions on rotavirus infectivity (Figure 2). Milk-fat globule membrane was a logical choice, bearing in mind the origin of lactadherin. Moreover, using Western blots (not shown) and ELISA (Table 1), lactadherin was shown to be a significant constituent of a commercially available bovine whey fraction containing high-molecular weight proteins and lipids (MMWP). Bovine MFGM was able to decrease the amount of RRV-infected MA104 cells, although it was difficult to keep the membranes in solution. A more pronounced effect was achieved with human MFGM, which resulted in only 9% infected cells at a protein concentration of 0.5 mg/

Table 1. Quantification of lactadherin and MUC1 in different bovine milk samples. Amounts are determined as described in the Materials and Methods section. Results are given as average values ($n = 4$) and weight percentage.

Milk fraction	Lactadherin (% wt)	MUC1 (% wt)
Raw	0.37	0.02
Skimmed	0.17	0.01
MFGM ¹	9.80	1.21
Whey (yellow cheese)	0.41	0.09
MMWP ²	0.17	0.32

¹MFGM = Milk fat globule membrane.

²MMWP = Macromolecular whey proteins.

mL, compared with the control experiments. The same level of inhibition could be reached using MMWP, but this material was more potent, reaching a high degree of inhibition at a lower protein concentration (significant at 0.03 mg/mL).

Accordingly, it seemed logical to test the ability of pure bovine lactadherin to affect RRV-infection of the MA104 cells. However, this protein proved to be without any measurable effect (Figure 3). Additional experiments with bovine lactadherin against 3 other rotavirus strains [nar3 (simian), YM (porcine), and RF (bovine)] confirmed the observed lack of antiviral properties (results not shown). Therefore, our focus shifted towards other potentially interesting bovine proteins present in MFGM and MMWP. A complementary survey of the effects of bovine MUC1 and lactoferrin was performed. In comparison with MMWP, MUC1 turned out to be

even more efficient, decreasing the infectivity rate significantly to 37.5% at only 6.3 μ g of protein/mL. Finally, no inhibitory effect was detected when bovine lactoferrin was added.

Human Lactadherin Inhibits Rotavirus Infectivity

Using a human intestinal cell line (Caco-2), we observed that human lactadherin (in contrast to the bovine ortholog) efficiently reduced the infectivity of the human rotavirus strain Wa (Figure 4). Human lactadherin and bovine MMWP demonstrated the same degree of inhibition (significant at 0.02 mg/mL). In both cases, a simple linear regression analysis demonstrated logarithmic linearity between the added protein and the infectivity efficiency with the same slope. Again, after changing to the Caco-2 cell line and Wa virus, bovine lactadherin failed to demonstrate antiviral activity, and MMWP still proved effective. Bovine MUC1 did not affect infection with Wa virus at the concentrations used, whereas the infectivity rate of the RRV virus was decreased by bovine MUC1, but not as efficiently as when MA104 cells were used.

Human Lactadherin Acts Before Cell Infection

The inhibitory mechanisms used by human lactadherin and bovine MMWP were studied by specially designed binding and infection assays. In the first 2 types of assays, protein/virus or protein/cell preincubations were performed before cell infection to evaluate at what stage interaction takes part. A third type of assay was carried out to see if the inhibitor acts at a postbinding level. This postattachment assay benefits from the fact that the virus, upon preincubation at 4°C, only binds to the cell, without penetrating and infecting the host cell. Human lactadherin only demonstrated inhibition when the virus and protein were preincubated before infection, whereas MMWP inhibited infection in the protein/virus preincubation and in the postattachment assay (Figure 5).

Figure 3. Effects of individual bovine proteins on RRV rotavirus infectivity of MA104 cells in relation to controls (no protein): Bovine MUC1; bovine lactadherin; and bovine lactoferrin. Results are presented as mean $\pm$ standard deviation of duplicates or triplicates. First point at which a statistically significant ($P < 0.05$) effect was obtained is indicated with an asterisk (*).

DISCUSSION

Both in vitro and clinical studies have suggested that human lactadherin possesses antirotavirus activity. A binding assay performed by Yolken and coworkers implied that lactadherin had an increased ability to bind rotavirus, and their results suggested that lactadherin could be a major inhibitor of rotavirus infections (Yolken et al., 1992). The following clinical study indicated an increased risk for getting a symptomatic rotavirus infection for breast-fed infants when the lactadherin concentration was low in the milk they received (New-

Figure 4. Dose-response of different milk fractions on Wa and RRV rotavirus infectivity of Caco-2 cells in comparison with controls (no protein). Protein was mixed with virus before infection. Bar fill patterns from left: Human lactadherin (horizontal dashed lines); bovine lactadherin (gray); macromolecular whey proteins (MMWP) (black); bovine MUC1 (white) on Wa infectivity; bovine MUC1 and RRV infectivity (hatched). Results are mean $\pm$ standard deviation of triplicates. Statistically significant ($P < 0.05$) effects are marked with an asterisk (*).

burg et al., 1998). The present study demonstrates for the first time that human lactadherin possesses rotavirus inhibitory activity, as it significantly reduced rotavirus infections in vitro in a test system using cells and viruses of human origin.

In human milk, the concentration of lactadherin peaks just after the postpartum period, when it is estimated to be 0.139 mg/mL, and it declines thereafter to approximately 0.066 mg/mL (Peterson et al., 1998). A significant inhibition of rotavirus infection was obtained below the physiological level used in the in vitro assay (about 50% reduction at 0.02 mg of lactadherin/mL, Figure 4). The amount of lactadherin in bovine milk was found to be within a comparable range (Table 1). However, pure bovine lactadherin showed no sign of inhibitory activity against any of the tested viruses or cell types. This result is not in agreement with that of Kanamaru and coworkers, who suggested that the rotavirus inhibitory effect of a bovine whey protein fraction could be caused by lactadherin (Kanamaru et al., 1999). It is not clear why human lactadherin, contrary

to the bovine protein, shows antirotaviral activity. The differences in the purification procedures used might influence the stability of the 2 proteins. However, bovine lactadherin obtained by the applied purification procedure has previously been shown to be functionally active (Andersen et al., 1997; Andersen et al., 2000; Shi and Gilbert, 2003). The sample of human lactadherin used in the present experiments was not totally pure. Thus, the observed differences in antiviral behavior might be related to the residual components. However, these impurities would have to be extremely potent, as they only account for about 5% of the total protein. One might speculate that the differences between human and bovine lactadherin are related to variations in the attached carbohydrates, or to structural differences. It is noteworthy that all glycosylations found in the N-terminal region of bovine lactadherin were located in the first EGF-domain, which is the domain absent in the human counterpart. Unfortunately, the glycosylation pattern and carbohydrate composition of human lactadherin remains to be established. Regardless, exam-

Figure 5. Investigation of inhibitory mechanism used by human lactadherin (left) and MMWP (right) on Wa rotavirus infectivity of Caco-2 cells in comparison with controls (no protein). Three different assays were performed: Virus/protein preincubation (white bars), virus/cell preincubation (black bars), and protein/cell preincubation (hatched bars). Results are given as mean $\pm$ standard deviation of triplicates. Statistically significant ($P < 0.05$) effects are marked with an asterisk (*).

ples have been given for large differences between structure and composition of glycans found in orthologous bovine and human glycoproteins from milk (e.g., Pallesen et al., 2001).

Evaluation of the inhibitory effect of human lactadherin by different types of binding and infection assays implies that the protein might function as a decoy. Preincubation of virus and lactadherin significantly reduced Wa rotavirus infection, suggesting that lactadherin reduces infection by hindering the attachment of the virus to the host cell. Furthermore, in the protein/cell preincubation assay, lactadherin failed to demonstrate any antiviral effect, suggesting that interaction with cell surface components is unimportant.

Although no antiviral activity could be attributed to bovine lactadherin, the study demonstrates the presence of bovine milk-derived inhibitory components in MFGM and MMWP. Based on observations by other investigators, the bioactivity of these more crude fractions might be attributed to mucinous proteins (Yolken

et al., 1992; Kanamaru et al., 1999) or lactoferrin (Superti et al., 1997, 2001). The bovine mucin MUC1 is among the major components present in MFGM (Pallesen et al., 2001) and MMWP (Figure 1B, lane 9 and Table 1). The present study demonstrates that bovine MUC1 is capable of reducing infection of the neuraminidase-sensitive RRV strain, whereas the neuraminidase-resistant Wa infection process is apparently unaffected by the presence of MUC1. These observations are in accordance with previous results with other heavily sialylated proteins (e.g., Yolken et al., 1994). Meanwhile, the present study illustrates that MUC1 cannot be the only active component in MMWP, as bovine MUC1 did not affect the action of the Wa virus.

That human and bovine MFGM preparations were able to inhibit propagation of RRV in MA104 is expected, because MUC1 and lactadherin are prominent constituents of these milk fractions. Besides the observed inhibitory abilities of human lactadherin, one might speculate that the relatively high content and

size of MUC1 in human milk (Mather, 2000) explains why human MFGM is a more effective inhibitor compared with the bovine equivalent.

Immunological methods have illustrated that bovine lactoferrin is among the constituents of MFGM and MMWP (results not shown). Antirotaviral activity might be attributed to this component, as experiments with the human colon adenocarcinoma cell line, HT-29, have indicated that bovine lactoferrin inhibits infection with the simian neuraminidase-sensitive rotavirus strain SA11 (Superti et al., 1997, 2001). Our experiments failed to show any inhibitory activity of bovine lactoferrin using the RRV/MA104 system, or when Wa virus and Caco-2 cells were used (results not shown). The latter is in accordance with results obtained by other researchers (Grover et al., 1997; Kanamaru et al., 1999). It is not evident why these studies reached such different conclusions. There are variations in the cell lines and virus strains used. Furthermore, the application of different assays might influence the obtained results. In the experiments with the SA11/HT-29 system, an indirect cell viability fluorescence assay was used, whereas our data were obtained by immunological detection of virus-infected cells.

Finally, it is interesting to observe that MMWP is an efficient inhibitor of rotavirus, reducing the infectivity by approximately 90% at a concentration of 0.5 mg/mL. The inhibitory activity of MMWP turns out to be very versatile. It was working against RRV in the MA104 assay, and it was equally effective in preventing infection of Caco-2 cells by 4 strains derived from humans and livestock animals (Wa, RRV, YM, and RF; results of the latter 3 not shown). This is an important feature, because rotavirus strains are not restricted to infecting only the species from which they were originally isolated. It appears that the inhibitory mechanism of MMWP involves virus interactions. Arguments come from preincubation experiments with MMWP and Wa rotavirus, showing efficient inhibition of the infection. The postattachment assay demonstrated that MMWP could inhibit infection of preattached virus, which is an interesting feature. This result indicates that MMWP is capable of interfering with the infection process even after virus-host cell interactions have been established. Analysis by SDS-PAGE (with and without reductive substances) illustrates the presence of IgG in MMWP (band at ~150 kDa in lane 8, Figure 1B). It has been shown that both raw and pasteurized milk contain detectable amounts of antibodies directed against rotavirus (Yolken et al., 1985). Taken together, this raises the question whether intrinsic IgG contributes to the antiviral activity of MMWP. However, Kanamaru and coworkers (1999) suggested that the inhibitory activity in whey was associated with components with molecu-

lar weight between 1 and 80 kDa, thus excluding IgG. It will be interesting to characterize the basis for the action of the MMWP fraction. Forthcoming experiments will have to clarify this.

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