The Bovine Lactophorin C-Terminal Fragment and PAS6/7 Were Both Potent in the Inhibition of Human Rotavirus Replication in Cultured Epithelial Cells and the Prevention of Experimental Gastroenteritis

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Rotaviruses are the leading cause of severe dehydrating diarrhea in children worldwide. We have found that high-MW glycoprotein fraction (F1) from cow’s milk whey has potent inhibitory activity against human rotavirus (HRV) in cell culture. The present study was undertaken to identify and characterize the components responsible for this inhibitory activity. F1 was initially heated at 95°C for 30 min, rendering milk antibodies inert, subjected to ammonium sulfate fractionation, and then resolved by two-dimensional polyacrylamide gel electrophoresis. After electroelution, we found that a heat-stable milk protein lactophorin C-terminal fragment (LP16) and bovine milk fat globule membrane protein PAS6/7 strongly inhibited the replication of HRV MO strains in MA104 cells. Furthermore, we found that prophylactic oral administration of F1 once before inoculation of the HRV MO strain obviously prevented the development of diarrhea in vivo. These non-immunoglobulin components are a promising candidate for a prophylactic food additive against HRV infection.

Key words: human rotavirus; diarrhea; bovine milk; lactophorin; PAS6/7

Rotavirus infection is the most important agent of severe gastroenteritis. It causes more than 527,000 deaths in children aged <5 years old from rotavirus diarrhea per year worldwide, with >85% of these deaths occurring in developing countries.1) The development of a new strategy to protect against infection and relieve the symptoms caused by rotaviruses must be of priority because of the high medical burden and human costs. In general, vaccination is most effective for protection against virus infection, and two rotavirus vaccines have been developed and approved in certain countries,1) but administration of the vaccine is strictly limited to infants aged between 6 and 12 weeks of age, and catch-up immunization is discouraged to reduce the risk of intussusception. Thus there is a need for alternative prophylactic procedures. In addition, in the case of immune-compromised hosts, especially infants and young children, alternative therapeutic approaches are needed.

One type of anti-virus agents is antibodies. It has been proposed that passive protection against rotavirus infection can be achieved by using anti-rotavirus antibodies (e.g., immunoglobulin G from colostrum of cows5,6) or immunoglobulin Y from hen egg yolks6,7) hyper-immunized with rotavirus).

On the other hand, studies of non-immunoglobulin components against rotavirus have also been reported. Supplemental dietary whey protein concentrates (WPC)6,7) and macromolecular bovine whey protein fraction (MMWP)8,9) were effective in protecting against simian rotavirus7) and murine rotavirus6,9) infection in vivo. Bojsen et al.9) found that a component of the MMWP fraction, mucin 1 (MUC1), besides immunoglobulins, have major anti-rotavirus capacity.

Human strains of rotaviruses are much different from those of animal origin, in that the presence of sialic acid is not essential for attachment to the surface of target cells.10) Thus, where the protection of children against rotavirus diarrhea is an issue, experiments using human rotavirus (HRV) are likely to produce more clinically relevant results. We have reported that the high-MW glycoprotein fraction (F1) of cow’s milk whey was potent in inhibiting the replication of HRV infection in vitro11) We failed to identify the components responsible for this anti-rotavirus activity, although we...
indicated the possible involvement of components smaller than 80 kDa. The aim of the present study was to identify and characterize the components inhibiting HRV infection in F1.

Materials and Methods

Cells and viruses. MA104 cells (a cell line from the rhesus monkey kidney) were cultured in Eagle’s minimal essential medium supplemented with 10% fetal calf serum. HRV strain MO (serotype G3P[8]) was propagated, and the virus titer was determined by fluorescent cell focus-forming units (FCFU) assay, as described previously.14) Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG were from Bio-Rad (Hercules, CA).

Materials. All chemicals used were of reagent grade. Bovine normal milk was collected from healthy Holstein-Friesian cows held at the Gifu University Farm. Colostrum whey from a cow hyper-immunized with HRV (rata whey) was prepared as described previously.21) Bovine lactoferrin was prepared as described previously.2) Rabbit polyclonal antiserum specific for bovine milk fat globule membrane (MFGM) protein PAS6/7 was prepared by Meiji Dairies (Kanagawa, Japan). Mouse monoclonal antibody specific for bovine lactoferrin (1C10) was prepared by immunizing mice with bovine MFGM.23) Mouse monoclonal antibody against pig rotavirus PO-13, used in virus replication inhibition (neutralization) assays, was prepared as reported previously.14) Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG was purchased from American Qualex (San Clemente, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were from Bio-Rad (Hercules, CA).

Preparation and fractionation of high-M, glycoprotein fraction (F1). Preparation of F1 was carried out basically according to the procedure described previously.11) Briefly, normal cow’s milk was separated into skim milk and cream by centrifugation at 1,500 × g for 20 min at 4°C. Caseins and whey proteins were prepared by isoelectric precipitation (pH 4.6) from the skim milk.

The whey proteins were rehydrated pH 6.0 in the resulting acid whey. High molecular weight whey protein concentrate (HWPC) was prepared by ultrafiltration/diafiltration of whey proteins using membrane cassettes with a nominal M, cut off of 3,000 kDa (Tosoh, Tokyo), followed by dialysis against distilled deionized water, and then freeze-dried. HWPC was put on a Sephacryl S-500 column (6 × 50 cm, GE Healthcare, Chalfont St. Giles, UK) in 0.05 M Tris–HCl buffer dissolved in and dialyzed against distilled deionized water, and then freeze-dried. HWPC was put on a Sephacryl S-500 column (6 × 50 cm, GE Healthcare, Chalfont St. Giles, UK) in 0.05 M Tris–HCl buffer containing 0.15 M NaCl, 1 m M Na3C03 (pH 8.0). The slightly opalescent eluate-containing substances excluded by the resin were collected, and are referred to as the high-M, glycoprotein fraction, F1.

F1 at 3 mg/ml in phosphate buffered saline (PBS) was heated at 95°C for 30 min and then cooled to room temperature. Denatured proteins were removed by centrifugation at 5,000 × g for 30 min. The supernatant fraction was freeze-dried after dialysis against distilled deionized water. The heated F1 was dissolved in PBS to give 3 mg/ml, and was added solid ammonium sulfate to give 55% saturation. The supernatant was collected by centrifugation at 7,000 × g for 30 min, and solid ammonium sulfate was added to give 90% saturation. The precipitate was collected by centrifugation at 7,000 × g for 30 min and dissolved in and dialyzed against distilled deionized water, and then freeze-dried.

Preparation of crude-PAS6/7 fraction. Preparation of PAS6/7 was done by the method of Kim et al.,15) with slight modifications. Briefly, butter milk and butter serum were obtained from the normal cow’s milk cream fraction by a conventional churning procedure. The released membrane fragments were separated by centrifugation at 3,000 × g and left to stand at pH 4.8 for 30 min, MFGM was isolated by centrifugation at 40,000 × g. Apo-MFGM was prepared by deolipidizing MFGM with a mixture of chloroform-methanol and subjected to size-exclusion separation on Sephacryl S-200 (GE Healthcare) to give crude PAS6/7 fraction.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and immunoblotting. A milk sample (approximately 150 μg) was dissolved in sample buffer, and then an IPG strip (Ready Strip, Bio-Rad) was caused to swell in PROTEIN IEF Cell (Bio-Rad) following the to manufacturer’s instructions. The second dimension was performed on 15% SDS–PAGE gels by the PROTEAN2 System (Bio-Rad), as described by Laemmli.16) After running, the gel was stained with Coomassie Brilliant Blue R-250 to visualize the proteins. For immunoblotting, separated proteins were blotted onto a polyvinylidene difluoride membrane (PVDF; Millipore, Bedford, MA) using Mini Trans-Blot Cell (Bio-Rad), as described by Towbin et al.,17) and visualized using specific antibodies and horseradish peroxidase-labeled secondary antibodies. The reacted proteins were visualized with aminobenzidine.

Electroelution from 2D-PAGE gel. This was done using Centrifor micro-electroeluter (Millipore) the manufacturer’s instructions at 200V for 4 h in elution buffer (25 mM Tris, 192 mM glycine, 0.02% SDS, 20% methanol), then concentrated and replaced by PBS in a Centricon YM-10 (M, cut-off, 10,000 kDa).

N-Terminal amino acid analysis. This was done by Takara Bio (Kusatsu, Japan) using the Edman degradation procedure (Code no. 6420).

Rotavirus replication inhibition (neutralization) assay. A replica- tion inhibition assay for HRV MO strain was carried out using MA104 cells, as described previously.17) A suspension containing infectious virus at a titer of 108–109 FCFU/ml was treated with 10 μg/ml trypsin (Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C. After appropriate dilution with Eagle’s minimal essential medium containing 10% fetal calf serum to give a titer of approximately 4 × 105 FCFU per 100 μl, aliquots were mixed with equal volumes (100 μl) of one-half serially diluted samples in microtubes for 1 h at 37°C. The diluted mixtures were then used to inoculate MA104 cells (2 × 105 cells/ml, 200 μl), and 20-μl aliquots of each were placed into the wells of a 24-well heavy Teflon- (HT) coated slide (AR Brown, Tokyo). The control gave about 100 infected foci per well without the test samples of milk. The cells were further cultured for 22 h at 37°C under an atmosphere of 5% CO2, and then fixed with cold acetone for 20 min. The foci numbers of infected cells were measured by indirect immunofluorescence assay using PO-13 monoclonal anti-pig rotavirus antibody16) and FITC-conjugated goat anti-mouse IgG serum. Neutralizing activity was expressed as the percentage reduction in the foci numbers of infected cells as observed by fluorescence microscopy. The minimum inhibitory concentration (MIC), the concentration showing 50% reduction of infected cells, was calculated for each sample from a logarithmic regression of the concentration-dependent percentage focus reduction.

Mouse diarrhea model. We used a previously developed mouse model of rotavirus gastroenteritis to study protective efficacy on the development of diarrhea.46) Pregnant BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). Litters of 5-7-old mice were orally inoculated with a single dose of 1 × 106 FCFU/50 μl of the MO strain by gavage. Stools were examined daily for the development of diarrhea by gentle abdominal palpation beginning at 1 d after inoculation for 3 d. Observation of muddy-mucus or liquid-mucus yellow stool yielded a judgment of diarrhea. To assess the effects of F1 in the prevention of HRV-induced diarrhea, suckling mice were given orally a single dose of 2.5 mg in PBS (50 μl) of F1 every 60 min before inoculation with HRV. Control mice were given PBS (pH 7.2). The care and experimental procedures were approved by the Animal Care and Use Committee of Gifu University.

Protein determination. The protein concentration was determined with a Bradford Protein Assay Kit (Bio-Rad) using bovine serum albumin as standard the manufacturer’s instructions.

Results and Discussion

The Lactophorin C-terminal fragment was identified as the component exhibiting potent anti-rotavirus activity. F1 was found to be highly potent in inhibiting replication of HRV with a MIC of 3.6 μg/ml (Fig. 1), several times higher than that of HWPC. As a positive
control, the whey from colostrum of cows hyper-immunized with HRV (rota whey) exhibited strong inhibitory activity against HRV, with a MIC of 0.15 mg/ml (Fig. 1). Lactoferrin from bovine milk exhibited only weak inhibitory activity, with a MIC of 230 mg/ml (Fig. 1).

We have found that F1 contains appreciable amounts of milk immunoglobulins. In order to eliminate their contribution to the anti-virus activity of F1, heat treatment was carried out. After incubation of F1 for 30 min at 95°C, the heated F1 remained highly inhibitory or became stronger in inhibiting replication of the virus, with a MIC of 0.91 μg/ml (Fig. 1). When the heated F1 was fractionated further with ammonium sulfate, the precipitate at 90% saturation of the soluble supernatant at 55% saturation (the 55–90% ppt) showed activity slightly higher than that observed before fractionation. The MIC of the 55–90% ppt was 0.68 μg/ml (Fig. 1).

To identify the components responsible for anti-HRV activity, we attempted to resolve protein components in the 55–90% ppt by 2D-PAGE, as shown in Fig. 2. Several spots observed were excised from the gels and collected by electroelution from 2D-PAGE. Among these, components with a molecular size of 16 kDa (noted in the boxed area in Fig. 2) exhibited rather high inhibitory activity against HRV replication, with a MIC of 0.016 μg/ml (Fig. 1). Sequencing analysis of the substance, indicated by an arrow in Fig. 2, yielded the N-terminal amino acids of ILKEKHL, which is consistent with the sequence of residues 69–75 of bovine milk heat stable lactophorin (LP). We refer this component as LP16 hereinafter. When we carried out immunoblotting of F1 using mouse monoclonal anti-LP antibody 1C10, spots corresponding to LP16 were clearly observed (data not shown).

One of the striking findings as to the active anti-HRV component is its extraordinary stability against heat. LP was originally found in the bovine milk heat stable proteose peptone (PP) fraction, and was referred to as PP component 3. There is a review regarding the molecular and functional characteristics of LP. LP consists of two major glycopeptides, of 28 kDa (LP28) and 18 kDa (LP18), with a variety of molecular forms. LP18 is thought to occur as a proteolytic degradation product of LP28 and to have one N-glycan and one O-glycan. The occurrence of LP16 in F1 might also be ascribed to proteolysis of LP28, and both glycans are expected to occur in the molecule. Hence it might be postulated, the profound heat-resistant character of its activity, that the inhibitory activity of LP16 could be attributed to its bound carbohydrates.

The final MIC of LP16 was found to be unexpectedly high, about 10 times higher than that of rota whey. The reasons for this high efficiency are unclear, but may not involve SDS remaining in the electroeluted LP16. However, because SDS has been reported to inhibit rotavirus infection by acting on viruses so as to modify the viral capsid and inactivate them, it is possible that SDS contributes to the anti-virus activity of LP16. Consequently, we investigated HRV infection under experimental conditions without SDS and then observed high inhibitory activity as shown in the heated F1 and the 55–90% ppt (Fig. 1), suggesting that SDS must not have pivotal role to anti-HRV activity. Furthermore, a preliminary experiment showed that the LP-rich fraction of PP resulted in as potent inhibition of HRV infection as F1 (data not shown). Hence we concluded that LP16 is a major inhibitory component of HRV infection.
PAS6/7 as the active component in inhibiting rotavirus replication

Previous studies by Yolken et al. indicate that the 46-kDa mucin-associated glycoprotein, lactadherin, in human milk is a major inhibitor of rotavirus infection. Accordingly, we examined for the presence of PAS6/7 in F1, the countercomponent of lactadherin in bovine milk, by immunoblot analysis with specific polyclonal antiserum. The results clearly demonstrated its involvement in F1, as shown in Fig. 3, though the level was too low to isolate it substantially for a neutralization assay. Hence we prepared a crude PAS6/7 fraction from the soluble fraction of bovine MFGM by the method of Kim et al. Crude PAS6/7 was to be potent in inhibiting the replication of HRV, with a MIC of 3.9 μg/ml (Fig. 4). Final purification of PAS6/7 was again accomplished by electroelution from 2D-PAGE gels. The purified-PAS6/7 exhibited inhibitory activity with a MIC of 1.8 μg/ml, as shown in Fig. 4.

PAS6/7 is known to be a major milk MFGM constituent, consisting of two N-terminal EGF domains followed by two repeated C domains with homology to the C1 and C2 domains of blood clotting factors V and VIII. We found that PAS6/7 was also potent in inhibition of the replication of HRV in vitro. However, our observations conflicted previous results reported by Kvistgaard et al. No anti-virus activity was found for PAS6/7 in their work. This discrepancy might be attributable to the difference in the methodology used in virus neutralization assay; in Kvistgaard’s protocol, after temporal incubation for 1 h of a virus-sample mixture with cells, the inocula were removed from the culture, whereas in this study virus and sample were present with cells together throughout the infection process. It has been reported that PAS6/7 binds integrin αvβ3 on MA104 cells, which is known as a cell-receptor for rotavirus. Prolonged incubation with PAS6/7 of MA104 cells might render the cell receptor inaccessible to the virus, resulting in virus neutralization. Further studies ought to be carried out to precise elucidation of the mechanism by which HRV infection of MA104 cells is inhibited by PAS6/7.

F1 protected suckling mice against HRV infection

Finally, to assess the protective efficacy of F1 against HRV-induced diarrhea, litters of 5-d-old suckling mice were given orally a single dose of 2.5 mg in PBS (50 μl) of F1 for 60 min before inoculation with HRV MO strain. Control mice were given PBS.

Two d after inoculation of HRV, seven of eight mice developed diarrhea in the PBS group, and two of eight mice did not recover from diarrhea even 3 d after inoculation. In contrast, none of the six mice developed diarrhea in the F1 group throughout the experimental period (Fig. 5). Under the same experimental conditions, a single dose of rota whey at 0.25 mg in 50 μl of PBS, a concentration ten times lower than that for F1, completely prevented diarrhea in suckling mice (data not shown).

In this study, unheated F1 was used as an orally administered sample in the experimental animal model. As mentioned above, F1 contained milk immunoglobulins, which might have caused anti-virus activity in this...
experimental gastroenteritis model, but heating at 95 °C for 30 min did not affect the anti-virus activity of F1 at all in the neutralization assay (Fig. 1). Therefore, the preventive efficacy of F1 against HRV gastroenteritis can largely be attributed to LP16 and PAS6/7.

In conclusion, a bovine milk fraction containing non-immunoglobulin components LP16 and PAS6/7 is promising as a prophylactic food additive against HRV infection.

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