Preliminary Communication

A High-Mr Glycoprotein Fraction from Cow’s Milk Potent in Inhibiting Replication of Human Rotavirus in Vitro

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Rotavirus is the major cause of infectious diarrhea in infants and young children all over the world. We have found that a high-Mr, glycoprotein fraction from cow’s milk is potent in inhibiting replication of human rotavirus in vitro. Since the activity seems to be unique and specific, this fraction may be useful as a novel agent for treatment or prevention of rotavirus diarrhea.

Key words: cow’s milk; milk mucin; glycoprotein; human rotavirus; neutralization

Diarrheal disease is a major health problem responsible for high mortality and morbidity in developing countries, and an important cause of hospitalization in developed countries.1 Among etiologic agents, rotavirus is the major cause of infectious diarrhea in infants and young children all over the world.2,3 While development of rotavirus vaccine candidates has been reported,4 passive protection could have advantages over vaccine owing largely to the following reasons: 1) the immune system in young infants is immature and they are incapable of active production of adequate amounts of intestinal antibodies; 2) there is insufficient understanding of how the virus causes disease and how immunity is generated.5 In addition, the elderly6,7 and the immunocompromised8,9 may be particularly prone to more severe symptoms when infected with rotavirus. Passive oral prophylaxis is thus of practical importance for the prevention of diarrhea due to infectious rotavirus.

Many of the studies involving passive protection against rotavirus diarrhea have been done using bovine colostrum or immunoglobulin concentrates prepared from bovine milk. In most cases positive results were obtained when the preparations used contained high levels of specific anti-rotavirus antibodies. These have been commonly produced by hyperimmunization of pregnant cows with certain rotavirus strains.10 We showed the practical significance of colostrum from hyperimmunized cows for the prevention of infectious diarrhea in a baby care center.11 Thus, the benefit of this type of passive prophylaxis is well-recognized. Its major limitation, however, is the poor feasibility of large-scale production of colostrum from hyperimmunized cows.

The critical initial event in rotavirus infection is attachment and entry of the virus into the target cells. Certain glycoconjugates on the surface of the target cells may serve as receptors for rotaviruses,12 though the chemical nature of the receptors has not yet been fully described. Non-immunological agents analogous to putative receptors (receptor analogues) have thus been explored for the ability to block viral attachment and to prevent infection.13-16 However, it is noteworthy that human strains of rotaviruses are much different from those of animal origin in terms of recognition of receptors on the surface of target cells; the presence of sialic acid as a constituent of the receptor is not essential for the former but the binding of the latter to the receptor unequivocally requires this constituent acid sugar.17 Therefore, if protection of human beings is the issue, human strains should be used in experiments intended to evaluate the true efficacy of this type of compounds in prevention of rotavirus infection. In this respect, it is of interest that a macromolecular complex of human milk (human-milk mucin) inhibits replication of human rotavirus strains in tissue culture and prevents development of rotaviral gastroenteritis in an animal model of infection as shown by Yolen et al.,18 a 46-kDa glycoprotein component of the complex being responsible for the virus binding. Most recently, Newburg et al.19 published a longitudinal field study strongly supporting the viewpoint that this 46-kDa mucin-associated glycoprotein present in human milk, now referred to as lactadherin, is significant in protection of breast-fed infants against symptomatic rotavirus infection. This protection is totally independent of products of the secretory immune system. Their study demonstrated for the first time the clinical relevance in a human population of a human-milk glycoprotein that is effective in protection against diarrhea, and their findings justify a proposal concerning the possibility of establishing a novel class of therapeutic agents (receptor analogues) suitable for oral supplementation against rotavirus diarrhea.18 It would be of great value, therefore, if similar non-immunological compounds with potent inhibitory activity against human rotavirus could be found in quantitatively unlimited sources such as cow’s milk. Since we have been investigating the inhibitory activity of immunoglobulins in cow’s milk against human rotavirus,10 the antiviral activities of fractions of cow’s

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milk other than those containing immunoglobulins have also been examined for their potential benefit in passive protection. This report describes a high-M, glycoprotein fraction from cow's milk found to have potent activity in inhibiting replication of human rotavirus in vitro.

The high-M, whey protein concentrate (HWPC) was prepared from bovine whey by ultrafiltration/diafiltration using membranes with a nominal M, cut off of 1000 kDa (Tosoh Corporation, Japan). HWPC was then put on a column of Sepharose CL-2B (Pharmacia Biotech, Sweden) in 50 mM Tris-HCl buffer at pH 8.0, containing 0.15 M NaCl, 2 mM EDTA and 0.02% NaN₃, and four fractions were obtained (Fig. 1). The fractions were then examined for anti-rotavirus activity. Human rotaviruses were propagated in MA104 cells, an established cell line derived from rhesus monkey kidney. The infected cells were stained by an indirect fluorescence method using the rabbit antiserum prepared against pigeon rotavirus PO-13 and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG goat serum (Organon Teknika-Cappel, NC, U.S.A.). Virus titer is expressed as fluorescent cell forming units (FCFU)/mL. The anti-rotavirus activity of the fractions was assessed by an neutralization assay as follows: the suspension containing infectious virus at a titer of 10⁵-10⁶ FCFU/mL was treated with 10 μg/mL trypsin for 30 min at 37°C. After appropriate dilution with Eagle's minimum essential medium (MEM) to give a titer of approximately 4 × 10⁵ FCFU per 100 μL, aliquots were mixed with an equal volume (100 μL) of one-half serially diluted samples in a microtiter plate for 1 h at 37°C. The diluted mixtures were then used to inoculate confluent MA104 cells (2 × 10⁵ cells/mL) and 20-μL aliquots of each were placed into wells of a 24-well HT coating slide (Erie Scientific Co., NH, U.S.A.). The cells were further cultured for 60-72 h at 37°C under an atmosphere of 5% CO₂. The cells were then fixed with cold acetone for 20 min. FCFU were measured by an indirect immunofluorescence assay. Neutralizing activity is expressed as the minimum inhibitory concentration (MIC), i.e., the minimum concentration showing 50% reduction in FCFU per well under a fluorescence microscope.

An initial neutralization assay was done using Hochi, a human rotavirus strain serotype 4, to find whether the fractions prepared had neutralizing activity. As shown in the inset of Fig. 1, marked inhibitory activity was observed in F1 and F2 while F3 showed only weak activity and F4 had no significant level of anti-rotavirus activity. When examined by SDS-PAGE, F1 and F2 were found to have almost the same protein composition; when electrophoresed under non-reducing conditions several discrete bands were evident over a wide range of molecular weight after staining with Coomassie Brilliant Blue (CBB) and/or periodic acid-Schiff (PAS) reagent (Fig. 2; cf. reference 22). The prominent band at the top of the separating gel may represent the previously reported bovine high-M, mucin-like glycoprotein (HMG). Comparing the staining patterns in Fig. 2 with those reported previously,²⁰ the major PAS bands with 120-200 kDa may correspond to PAS-I, the major bovine milk fat globule membrane (MFGM) component, which is only poorly stained with CBB. Another major PAS band of 80 kDa could not be identified in this study and is referred to as 80 K. Other glycoproteins were found in F3 and F4, the major components being IgM and lac-

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**Fig. 1.** Fractionation of High-M, Whey Protein Concentrate (HWPC) by Gel Filtration on Sepharose CL-2B.

The column (5 x 80 cm) was equilibrated with 0.05 M Tris-HCl, pH 8.0, containing 0.15 M NaCl, 1 mM NaCl, 1 mM EDTA, and 0.02% NaN₃. Elution was done at a flow rate of 80 mL/h at 4°C. Ten-ML fractions were collected. The inset shows the minimum inhibitory concentration of each fraction against human rotavirus Hochi (serotype 4) as measured by a neutralization assay (details are given in the text).

**Fig. 2.** SDS-PAGE Profiles of F1 and F2.

Electrophoresis was done using 6% acrylamide at 30 mA for 4 h, followed by staining with Coomassie Brilliant Blue (CBB) or periodic acid-Schiff (PAS) reagent. The numbers in the right margin indicate the molecular mass (kDa) of the standard proteins (Bio-Rad): HMG, high-M, mucin-like glycoprotein; IgG, immunoglobulin G; Lf, lactoferrin; BSA, bovine serum albumin; Lph, lactophorin.
tophorin in the former and IgG, lactoferrin, and BSA in the latter (data not shown). The two major non-glycosylated bovine whey proteins, α-lactalbumin and β-lactoglobulin, had been previously removed from HWPC and only traces of these proteins could be detected by SDS-PAGE in any of the fractions. These results thus indicate that certain protein component(s) in the high-M₆ glycoprotein fraction from bovine milk is effective to inhibit replication of human rotavirus, while the major whey proteins including lactoferrin are almost inactive.

In view of its potent antiviral action, we then compared the inhibitory activity of bovine F1 with that of human milk. The human F1 was prepared by the same procedure, except that a membrane with a nominal M₈ cut off of 300 kDa were used in the ultrafiltration/diafiltration step. This fraction was previously found to contain predominantly MUC1 mucin (human-milk mucin), 24 with which lactadherin, a non-immunoglobulin component with anti-rotavirus activity, has been reported to associate. 18,25 Since prominent CBB staining was found at positions corresponding to immunoglobulins in bovine F1 (Fig. 2), we used an ELISA to measure their relative abundance and found that IgG indeed comprised about 10% of the total protein while the amounts of IgA and IgM were negligible. To eliminate the possibility that this type of immunoglobulins contribute to the inhibitory activity, the IgG was absorbed using a High-Trap protein G affinity column (Pharmacia Biotech), after which the IgG was found to be as low as 1 μg/mL, only 0.1% of the total protein in this fraction.

The results obtained from the neutralization assay using these preparations are summarized in Table 1, and the MIC values obtained for three representative human rotavirus strains (Wa of serotype 1, KUN of serotype 2, and Mo of serotype 3) and NCDV, a bovine rotavirus strain, are shown. Again, marked neutralizing activity was found in both milk-derived fractions against human rotavirus strains, showing MIC values as low as about 20 μg/mL. It can thus be concluded that bovine F1 has a strong inhibitory effect against all four human serotypes used in this study. Furthermore, the activity seems to be quite unique and specific. The activity is approximately 20–30 times greater than that of bovine MFGM, bovine submaxillary mucin, or ovomucin. Porcine stomach mucin was totally inert in this assay system. Interestingly, the bovine milk F1 was less active against bovine rotavirus strain NCDV than it was against the human rotavirus. In contrast, glycophorin derived from the erythrocyte membrane, although totally inactive against human rotavirus strains, was strongly inhibitory on the bovine strain, with an MIC several times lower than that of F1. These results are highly compatible with previous findings that animal rotaviruses, but not human strains, cause hemagglutination. 27

In conclusion, this investigation indicates that a high-M₆ glycoprotein fraction, easily prepared on a large-scale from cow’s milk, is potentially useful for the prevention of diarrhea caused by infectious human rotavirus. The precise chemical nature of the active component(s) in bovine F1 remains to be discovered. By analogy with lactadherin in human milk, PAS 6/7 (the component enzyme in bovine milk, also referred to as component 15/16, MFG-E8, or MGP57/53) 28 might be responsible for the activity; a preliminary fractionation by gel filtration using a dissociating buffer system containing SDS resulted in the finding that the inhibitory activity was associated with a low-M₆ fraction which contained neither HMGP, nor PAS-1, nor 80 K. Further studies focusing on purification and characterization of the active component(s) are now in progress. The final goal would be the worldwide use of bovine F1, and possibly the purified component(s), in prevention of diarrhea caused by human rotavirus.

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Table 1. Neutralizing Activity Against Human and Bovine Rotavirus of Various Glycoproteins

<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>Wa</th>
<th>KUN</th>
<th>MO</th>
<th>NCDV</th>
</tr>
</thead>
<tbody>
<tr>
<td>bovine F1 ⁶</td>
<td>17 ± 1</td>
<td>15</td>
<td>18 ± 4</td>
<td>120 ± 0</td>
</tr>
<tr>
<td>human F1 ⁶</td>
<td>23 ± 2</td>
<td>25</td>
<td>12 ± 0</td>
<td>ND</td>
</tr>
<tr>
<td>MFGM ⁶</td>
<td>258 ± 129</td>
<td>ND</td>
<td>404 ± 52</td>
<td>ND</td>
</tr>
<tr>
<td>BSGM ⁶</td>
<td>&gt; 374</td>
<td>ND</td>
<td>561 ± 187</td>
<td>73 ± 0</td>
</tr>
<tr>
<td>PSM ⁶</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>OVM ⁶</td>
<td>378 ± 3</td>
<td>ND</td>
<td>375 ± 0</td>
<td>281 ± 94</td>
</tr>
<tr>
<td>glycophorin ⁷</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>&lt; 50</td>
</tr>
</tbody>
</table>

¹ Activities are expressed as MIC (minimum inhibitory concentration, μg/mL), the lowest concentration showing a 50% reduction in FCPU per visual field. Viruses with titer of 10⁶ FCPU/mL were used.

² The results are expressed as the mean ± SD of duplicate determinations, except for KUN. MIC values greater than 1000 μg/mL are expressed as “>…”

⁴ Sepharose CL-2B void volume fraction of milk whey (see text).

⁵ Bovine milk fat globule membrane.

⁶ Bovine submaxillary gland mucin (Sigma Chemical Co.)

⁷ Porcine stomach mucin (Sigma Chemical Co.)

⁸ Ovomucin (Tatyo Chemical Co.)

⁹ Purchased from Sigma Chemical Co.

References


