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CHARACTERIZATION OF A PORCINE ENTEROCYTE RECEPTOR FOR GROUP A ROTAVIRUS

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SUMMARY

We have identified, purified to apparent homogeneity and chemically characterized a biologically-relevant porcine enterocyte receptor for group A porcine rotavirus. Ceramide glycanase digestion followed by acid hydrolysis and monosaccharide compositional analyses indicated the receptor is a family of two GM_3 gangliosides, one containing N-glycolyl-neuraminic acid and the other N-acetylneuraminic acid. Both gangliosides displayed dose-dependent inhibition of rotavirus binding to, and infectivity of, host cells. Inhibition of infectivity in a focus-forming-unit-reduction assay was achieved with as little as 2 nmols of $NeuGcGM_3$ (50% inhibition with 3.97 nmol) or $NeuAcGM_3$ (50% inhibition with 9.84 nmol) per 10^4 FFU of virus. Preliminary data suggest specific porcine GM_3 carbohydrate fine structure or spatial orientation of the sialyloligosaccharide epitopes of the $holoGM_3$ gangliosides may be crucial to enterocyte receptor recognition by rotavirus. We have quantified both $NeuGcGM_3$ and $NeuAcGM_3$ in enterocytes of various-aged pigs from newborn through 16 weeks and have found with increasing age the amount of both GM_3 derivatives, especially $NeuGcGM_3$ per gram (dry weight) intestinal brush border decreases rapidly from newborn through 4 weeks of age. These results may help explain the age-sensitivity of piglets to severe rotavirus diarrhea.

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INTRODUCTION

Rotaviruses are a major cause of viral diarrhea in the young of most mammalian species. Their significance in terms of individual mortality is well documented (Bohl, 1979; Kapikian and Chanock, 1990; Leece et al., 1978; Vesikari et al., 1975; Center for Disease Control, Morbidity and Mortality Report 41;1–20).. They are a major cause of morbidity in infants and young children in developed countries and of morbidity and mortality in developing countries. It is estimated that more than one million children die each year of human rotavirus infections (Bern et al., 1992; Blacklow and Greenberg, 1991). Rotavirus infections are also of prime agricultural importance since they frequently cause serious neonatal diarrheal diseases of many animal species, most importantly neonatal and post-weaning pigs and calves. Morbidity due to rotavirus infections in these species often reaches 80% while mortality can be as high as 60% (Bohl, 1979).

In spite of considerable research efforts in many laboratories, rotavirus diarrhea continues to be a serious and costly disease in both animals and man and has recently become important in causing significant illness in immunocompromised people (Saulsbury et al., 1980; Yolken et al., 1982) and debilitated neonates (Rotbart et al., 1988). Despite extensive efforts including the use of reassortants, attenuated live strains and vector expression of viral capsid proteins, a vaccine which is protective across all rotavirus serotypes is not available. In order to establish a firm scientific basis for the ultimate prevention and control of rotavirus disease a greater understanding of the molecular mechanisms of rotavirus host cell interaction are required.

The earliest and requisite step for productive viral infection is recognition and binding to villous tip enterocytes. The tissue and cell type specific tropisms displayed by rotaviruses is consistent with the hypothesis that a specific host cell-surface receptor(s) mediates recognition. Our recent work using a new binding assay to measure virus attachment to host cells in suspension resulted in the identification and partial purification of a porcine enterocyte ganglioside fraction that appears to function as an *in vivo* relevant cell-surface receptor for the homologous group A porcine rotavirus serotype (Rolsma et al., 1994). In the present report we review these findings and extend them to include demonstration of ganglioside-mediated inhibition of infectivity of MA104 cells in culture and preliminary structural characterization of its sialyloligosaccharide moiety.

MATERIALS AND METHODS

Cells and Virus

Group A (OSU) porcine rotavirus was propagated in MA-104 cells using modifications of standard techniques (Rolsma et al., 1994). Following extraction from cellular material, the virus was purified by isopycnic centrifugation on a cesium chloride gradient. Double- (ds) and single-shelled (ss) virus containing bands were collected and radioiodinated as previously described (Rolsma et al., 1994). Enterocytes for use in binding assays were harvested from the intestinal mucosa of 3 to 7 week old crossbred piglets as previously described (Rolsma et al., 1994).

Binding Assay

Binding of radiolabeled ds rotavirus to host cells in suspension was measured as previously described (Rolsma et al. 1994). Briefly, cells (MA 104 or enterocytes) were washed three times with MEM (without serum) by centrifugation at 200 x g and adjusted to a concentration of 2 x 10^6 cells/ml. Two ml of cells in MEM (assay buffer) were mixed with 10–30 µl (50 -150 ng) of [^{125}I]-ds or [^{125}I]-ss rotavirus and rotated end-over-end at 5 RPM. Aliquots were removed at appropriate time intervals and immediately overlaid onto 0.5 ml of a silicone /mineral oil mixture in 1.5 ml microcentrifuge tubes and centrifuged at 15,600 x g for 45 seconds in a microcentrifuge. Radioactivity in the pellets and supernatants was enumerated in a gamma counter and virus binding was defined as the pellet cpm divided by the sum of the supernatant and pellet cpm x 100.

Competitive Binding Assay

A variety of biomolecules typically found enriched on eukaryotic cell surfaces, particularly glycoconjugates, were evaluated for the ability to block rotavirus binding to host cells. Radiolabeled virus was preincubated with putative competitor molecules for 30 minutes prior to the addition of cells and virus binding measured following 30 min incubation as described above.

TLC Overlay Assay

Direct binding of [125-I] ds rotavirus to individual gangliosides was measured using modifications of a TLC blot overlay assay as previously described (Schnaar et al., 1989). Briefly, gangliosides were chromatographed on plastic-backed TLC plates , the plate thoroughly dried by air evaporation to remove solvent and then overlaid with 1x10^6 to 1 x 10^7 cpm of [125-I] ds rotavirus in 10 ml of PBS, pH 7.3 and allowed to incubate for 1 hour at room temperature. The plates were washed 3 times with PBS, and dried. Virus binding was visualized by standard autoradiography.

Focus Forming Infectivity Assay

A confluent monolayer of MA-104 cells in 24 well plates was rinsed twice with PBS. One ml of virus MEM was added to each well. Plates were incubated at 37°C in 5% CO_2 incubator for 3 hours. The $CsCl_2$ purified ds virus suspension was treated for 30 minutes at 37°C with 10 µl of 1 mg/ml crystallized trypsin per ml of virus suspension. Appropriate dilution of trypsinized virus suspension in virus MEM was performed to achieve a final dilution that resulted in 1000 ffu / 75 µl. Aliquots were treated with potential inhibitors or were sham treated at RT for 15 minutes. Following prechilling of all reagents and 24 well plates to 4°C, virus MEM was aspirated from the 24 well plates. Each well was inoculated with 75 µl of each virus dilution followed by incubation on ice at 4°C for 15 minutes. One ml of ice cold virus MEM was added, aspirated and replaced with 1 ml of fresh virus MEM. Plates were then warmed to 37°C and incubated at 37°C in a 5% CO_2 incubator for 16–18 hours. Virus infectivity was quantified using a peroxidase immunohistochemical assay.

Purification of Porcine Enterocyte Gangliosides

Extraction and purification of enterocyte gangliosides was performed using modifications of techniques previously described (Rolsma et al., 1994). Final separation of inhibitory monosialoganglioside species isolated from newborn pigs was accomplished using a semi-preparative Econosil HPLC column eluted with a linear gradient of chloroform/methanol/water beginning at 65:30:3 and ending with 60:35:8 (v/v/v). Final purity was analyzed by both HPTLC and HPLC.

Monosaccharide Compositional Analysis

Ganglioside neutral sugar composition were determined following trifluoroacetic acid hydrolysis by high performance anion-exchange chromatography under alkaline conditions using pulsed amperometric detection (HPAEC-PAD). Sialyloligosaccharides were released from purified porcine gangliosides by ceramide glycanase digestion [50–100 μg ganglioside (as sialic acid) were evaporated to dryness, resuspended in 50 μl 0.2M acetate, pH 5.0 to which was added 100 μl sodium taurodeoxycholate (2 mg/ml), 50 μl of sodium cholate (1.5 mg/ml) and 0.5 units ceramide glycanase (V-labs)] at 37 °C for 24 hr. Released oligosaccharides were then purified prior to monosaccharide analysis by C-18 reverse phase chromatography. Neutral sugar composition was quantified following trifluoroacetic acid hydrolysis using high pH anion-exchange HPLC chromatography and detection by pulsed amperometry [(HPAEC/PAD), Dionex CarboPac PA-1 column and a DX-300 chromatography system equipped with AI-450 chromatography workstation software] using modifications of previously described methods (Hayase et al., 1992) Standard curves for quantitation of individual monosaccharides were generated using 5 injections each of L-fucose, D-galactose, D-galactosamine, D-glucosamine, D-glucose and D-mannose over a range of 100 - 1000 pmoles. Sialic acid was quantified by HPLC as previously described (Powell and Hart, 1986) or by HPAEC/PAD using NeuNAc and NeuGc standards. Approximate monosaccharide molar ratios of NB1 and NB2 were calculated based on the quantity of galactose, glucose and sialic acid released from an identically treated GM3 standard. The molar ratios of each sugar obtained from NB1 and NB2 were calculated by dividing the unknown value by the value obtained for the respective monosaccharide detected in an identical amount of standard GM3 (1:1:1, sialic acid:galactose:glucose).

RESULTS

Binding Assay

Radioiodinated, infectious, double-shelled (ds) rotavirus binds rapidly and specifically to MA-104 cells and enterocytes in suspension whereas non-infectious, single-shelled (ss) rotavirus does not (Rolsma et al., 1994). Treatment of double-shelled virus with EDTA (converts ds to ss particles) or anti VP7 (outer capsid protein) blocks virus binding. The amount of double-shelled virus binding was proportional to the target cell and virus concentration used in the incubation. The observed difference in binding activity correlates with the differing biologic behavior of these two populations of virions and suggests the receptor that mediates this interaction has biologic significance.

Screening of Potential Receptor Molecules

In initial experiments designed to quickly gain insight as to the possible chemical nature of putative virus receptors, we screened numerous naturally occurring and synthetic glycoconjugates for their ability to compete for ds virus binding to both porcine enterocytes and MA-104 cells. Of all the glycoconjugates tested, a mixed bovine brain ganglioside fraction exhibited the greatest inhibitory effect and the inhibition was dependent on the amount of added ganglioside (Rolsma et al., 1994). Examination of individual gangliosides contained within the mixed ganglioside fraction revealed the inhibitory activity was specific for monosialogangliosides (Figure 1). These data also demonstrated that the inhibitory activity is markedly sensitive to subtle changes in the oligosaccharide portion of the ganglioside molecule. Of all the gangliosides tested, GM2 and GM3 were the most active and had comparable inhibitory activity. We previously demonstrated that the sialic acid moiety is required for the virus binding inhibitory activity of crude and partially purified porcine enterocyte gangliosides (Rolsma et al., 1994). Similarly, the asialoderivative of GM1 or GM2 (GA1 and GA2, respectively) failed to show substantial inhibitory activity. The presence of one or two additional sialic acid residues to GM1 (yielding GD1A and GD1B or GT1B respectively) also markedly reduced inhibitory activity. These effects

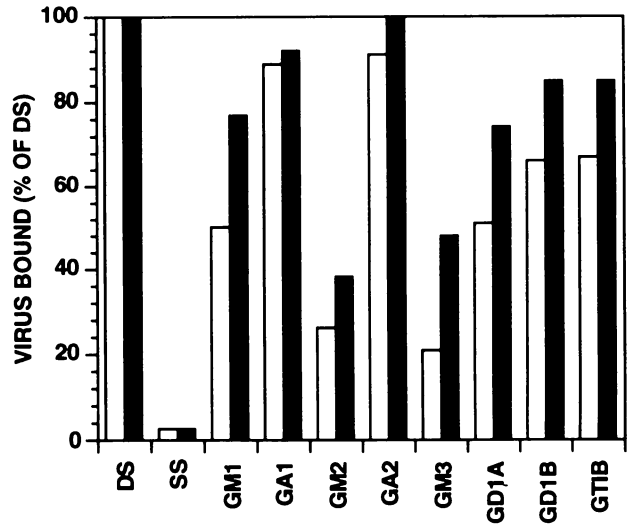

Figure 1. Effect of Various Gangliosides on Binding of Rotavirus to Porcine Enterocytes and MA-104 Cells. [125-I]-ds rotavirus (50 ng) was preincubated with the indicated gangliosides (10 μg) for 30 min, added to either porcine enterocytes (closed bars) or MA-104 cells (open bars) and virus binding measured using the competitive binding assay. Virus binding is expressed as the percentage of control (no ganglioside added) ds virus binding.

were also dose dependent (data not shown) and were seen when either MA-104 cells or porcine enterocytes were used in the blocking assay.

Purification of Porcine Enterocyte Gangliosides

Based upon the results obtained in the screening assays, fractionation of enterocyte gangliosides isolated from a susceptible piglet led to the isolation of a family of naturally occurring monosialogangliosides which demonstrate receptor-like inhibitory activity(Rolsma et al., 1994). This monosialylganglioside containing fraction obtained from a pooled extract of newborn piglet intestine was further purified by a combination of solvent partitioning and preparative HPLC and TLC chromatography. This purification yielded two major monosialogangliosides, NB1 and NB2 which displayed TLC mobility between that of bovine standard GM3 and GM2 (Figure 2).

Both NB1 and NB2 chromatographed as single, resorcinol-positive bands on HPTLC following extensive extraction, solvent partitioning, TLC and HPLC. Furthermore, NB1 and NB2 chromatographed as single entities in two other solvent systems (data not shown). Each of these purified gangliosides displayed dose-dependent inhibition of ds rotavirus binding (Figure 2b). In addition, direct virus binding to purified NB1 was demonstrated using a TLC overlay assay (Figure 2b inset). These data suggest the inhibition of virus binding to host cells in the presence of exogenous porcine ganglioside, NB1, is the

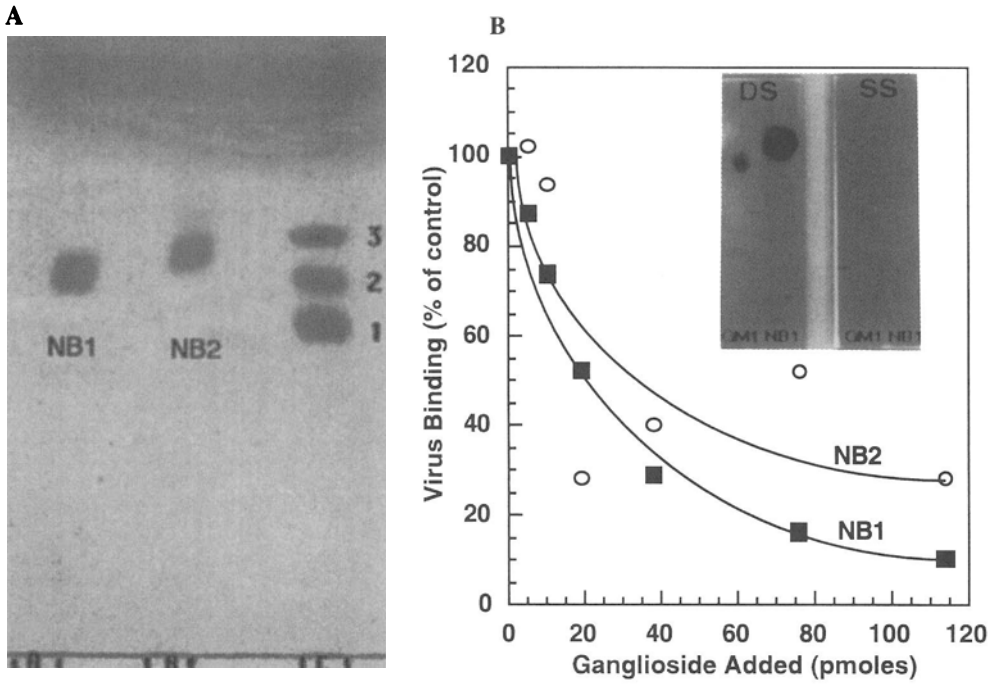

Figure 2. Effect of Purified Porcine Gangliosides on Binding of Rotavirus to MA-104 cells. A. Thin layer chromatography of NB1 and NB2. Equal quantities (2 nmoles (as sialic acid) of each were spotted onto a HPTLC plate and developed as described in Materials and Methods. Bovine standard gangliosides GM1, GM2 and GM3 are shown in the far right lane of the chromatograph. B. The effect of increasing amounts of NB1 and NB2 on binding of rotavirus. The ability of virus to directly bind to NB1 is shown in the inset. Virus binding to MA104 cells and to NB1 using TLC overlay was determined as described in Materials and Methods

result of a direct interaction between the ganglioside and ds rotavirus rather than due to secondary effects of the ganglioside on host cells. The similar mobilities of NB1 and NB2 suggested only minor structural differences may exist between these two gangliosides perhaps representing differences in monosaccharide linkages and/or type of sialic acid moiety present.

Ganglioside-Mediated Inhibition of Infectivity

Increasing concentrations of enterocyte NB1, NB2 and bovine brain GM3 were evaluated for their ability to inhibit porcine rotavirus infection of MA-104 cells. Both the NB2 and NB1 displayed dose-dependent inhibition of infectivity with NB1 being the most inhibitory (I_{50}= 4 nmoles NB1/1000 ffu virus). NB1 was approximately 2.5 times more inhibitory than NB2 and 5 times more than bovine brain GM_3.

Structural Characterization of NB1 and NB2

Effect of Pretreatment with Neuraminidase, Protease, Ceramide Glycanase and Heat. Treatment of enterocyte gangliosides with neuraminidase which removes sialic acid and ceramide glycanase which removes the oligosaccharide portion of the ganglioside from the lipid backbone reduces inhibitory activity by 80% and 35%, respectively. Boiling and exhaustive trypsin digestion (treatments designed to destroy protein structure) had no effect on inhibitory activity. These data along with the purification data strongly suggest that blocking activity is attributable to sialylated gangliosides.

Monosaccharide Compositional Analysis of NB1 and NB2. Monosaccharide compositional analysis of NB1 and NB2 by HPAEC-PAD was performed following release of oligosaccharides by ceramide glycanase and acid hydrolysis as described in Materials and Methods. During initial experiments aimed at optimizing hydrolysis conditions it was discovered that more accurate and consistent molar ratios for known ganglioside standards were obtained by releasing the entire oligosaccharide using ceramide glycanase prior to acid hydrolysis. Chromatography (HPAEC/PAD) of ceramide glycanase/trifluoroacetic acid hydrolysates revealed NB1 and NB2 each contain D-galactose and D-glucose as the only neutral sugar residues in amounts approximating those recovered from the GM3 standard. Similarly, both gangliosides contain sialic acid in quantities similar to standard GM3, however, the type of sialic acid detected in NB1 and NB2 differed. NB1 contains only NeuGc whereas NB2 contains the N-acetyl derivative (NeuNAc). Molar ratios calculated based on the results of GM3 analyses as described in Materials and Methods indicate the basic oligosaccharide unit in both NB1 and NB2 is sialyllactose and that NB1 and NB2 differ in only the type of sialic acid derivative they contain. These data are consistent with the NB1 and NB2 belonging to the GM3 class of ganglioside (Table 1).

Age-Related Changes in Porcine Enterocyte NB1 and NB2 Concentration. In most, if not all, species rotavirus morbidity and mortality are primarily seen in the young. Porcine rotavirus diarrhea displays a distinct age associated incidence, seen primarily in nursing piglets and during the immediate post-weaning period. Therefore, we investigated whether a correlation might exist between age-related incidence of natural rotavirus disease and the concentration of the two virus receptor gangliosides, NB1 and NB2, we identified. NB1 and NB2 were partially purified from newborn (colostrum deprived) and 1, 2, 3, 4, 5, 6, 8, 12 and 16 week old pigs and quantified by determination of the amount of

Table 1. Monosaccharide compositional analysis of NB1 and NB2

	Fuc	GalNAc	GlcNAc	Gal	Glu	Man	NeuNA	NeuGc
NB1	0	0	0	1040	1395	0	0	107
NB2	0	0	0	701	1658	0	116	0
GM3	0	0	0	1039	1389	0	141	0

Monosaccharide compositional analysis of NB1 and NB2 were performed as described in Materials and Methods. Amounts of each monosaccharide detected are listed in pmoles.

NeuGc (NB1) and NeuNAc (NB2). NB2 alone or with NB1 are the predominate gangliosides in porcine enterocyte extracts of all age groups examined constituting nearly 100% of the lipid-bound sialic acid present in enterocyte extracts. NB1 is found in high concentrations in newborn piglets contributing approximately 78% of the total monosialylganglioside. NB1 rapidly declines in concentration in the first 3–4 weeks of life from approximately 700 nmoles of ganglioside sialic acid per g dry weight of intestine in the newborn to about 100 nmoles/g in 4 week old pigs. NB2 is found in much lower concentration in the newborn (200 nmoles/g) than NB1 and decreases only gradually during the next 16 weeks. At 16 weeks the concentration of NB2 is still approximately 100 nmoles/g. The total NB1 and NB2 concentration decreases by 90% (900 to 100 nmoles/g) by 4 weeks of age at which time the NB1 and NB2 concentration are approximately equal. These data suggest the severity of disease seen in young experimentally infected piglets may be dependent on the enterocyte NB1 concentration.

DISCUSSION

Gangliosides are biologically active molecules which are only present in significant quantities in the outer leaflet of the lipid bilayer that comprises the cell membrane of eukaryotic cells. Our investigations into the mechanisms of rotaviral binding to host cells indicate that Group A porcine rotaviruses utilize porcine enterocyte monosialylgangliosides to specifically bind to host cells. Studies on the structure of the most active ganglioside, analysis of these gangliosides' involvement in infectivity and determination of age related changes in the ganglioside content of porcine enterocytes are consistent with an N-glycolylsialyllactose monosialylganglioside[N-glycolylGM$_3$ (NB1)] serving as a potent rotavirus receptor in pigs. Further experiments to define the fine carbohydrate and ceramide structural determinants as well as the possible topographical requirements necessary for receptor-activity are currently underway. Preliminary experiments indicate the isolated N-glycolylsialyllactose released from NB1, standard α2–3 and α2–6 sialyllactose do not inhibit rotavirus binding at 1–3 times the concentration of intact NB1. These results suggest positional or spatial arrangement of oligosaccharide epitopes are critical for virus binding and are consistent with the hypothesis that rotavirus-host cell recognition may be mediated by multivalent binding of virions to optimally-spaced low affinity cell surface carbohydrate epitopes which cumulatively result in a high affinity interaction. These experiments have expanded our understanding of early rotavirus host cell interactions which may lead to a more rational approach to development of novel, effective rotavirus preventatives such as inclusion of competitive inhibitors of rotaviral binding in porcine creep feeds.

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