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Characterisation of G serotype dependent non-antibody inhibitors of rotavirus in normal mouse serum

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Summary. Serotype specific (non-immunoglobulin) inhibitors of rotavirus have been identified in normal mouse serum obtained from BALB/c, CBA, and BL10 mice. Sialic acid was essential for the neutralising activity as sera treated with the neuraminidase from *Vibrio cholerae* failed to neutralise rotavirus. G serotypes 4, 5, 7, 8, 9, & 10 were unaffected by the inhibitor(s) while G serotypes 1, 2, 6 and two G3 strains were neutralised to significant titres. Assessment of neutralisation of reassortants suggested that VP7 is the virus protein involved in the interaction although it remains possible that VP7 is influencing VP4 binding. Analysis of the sera by Western blot followed by virus overlay confirmed that binding is dependent on the presence of sialic acid. The human strain tested, Wa, bound to two (glyco)proteins (50 & 80 kDa) while the bovine strains tested, NCDV and UK bound to one (55 kDa) and two (36 & 55 kDa) proteins respectively. This indicates that while the bovine rotaviruses may bind to a common element, the human strain binds to clearly distinct proteins. We propose that these inhibitors interact with animal rotaviruses in a manner analogous to that by which they attach to target cells. The glycoprotein to which NCDV bound was purified and identified by N-terminal sequencing as murine alpha-1-anti-trypsin (MuAAT) and was confirmed to possess both neutralisation and anti-trypsin activity. Since MuAAT is known to possess only three N-linked glycans, identification and analysis of the actual virus-binding structure should now be possible.

Introduction

Rotaviruses are recognised as a major cause of severe infantile gastroenteritis in a wide range of mammals, including man. The ubiquity of rotavirus, its impact on human health and the need for prevention or control of infection have been well documented [4]. In order to design new anti-viral strategies, the nature of the cell receptor and its interaction with the virus at a molecular level must be understood. The double-stranded RNA genome comprising eleven segments is

enclosed within an icosahedral double capsid [13]. The outer capsid consists of two proteins: the larger polypeptide, VP4, encoded by segment 4, and the glycosylated protein VP7 encoded by the 7th, 8th or 9th gene segment depending on the strain [7, 8]. Neutralisation of rotavirus infectivity has been shown using antibodies directed at either of the two surface proteins VP4 or VP7 [11, 14]. Extensive effort has gone into determining which of the two outer capsid proteins acts as the primary viral attachment protein. Earlier studies suggested VP7 as the more likely candidate. Neutralising monospecific antisera directed to VP7 of bovine and human rotaviruses were shown to block virus adsorption to MA104 cells [10, 23]. However, a more recent report by Ruggieri and Greenberg [34], showed that monoclonal antibodies directed at VP7 do not block virus atachment as efficiently as those directed at VP4. Ludert et al. [22] have recently shown VP4 to be the cell attachment protein by determining the infectivity phenotypes of VP4 monoreassortants on neuraminidase treated MA104 and Caco-2 cells. Furthermore, Bass et al. [1] have shown that baculovirus expressed VP4 binds to brush-border membranes and Crawford et al. [5] have reported the necessity for VP4 in virus-like particles for binding to MA104 cells. Recent cryoelectron microscopy of rotavirus shows VP4 to be a protruding protein on the surface of the virus particle, forming spikes which project from the outer shell [29]. Visualisation of the relative topology of VP4 and VP7 by such means suggests that VP4 is the polypeptide more likely to interact initially with the cell surface. Based on this collective evidence, it appears to be established that VP4 is the primary cell attachment protein of rotavirus.

Less clearly defined is the cellular receptor for rotavirus and thus the chemical nature of the interaction between the virus particle and the cell. Numerous recent reports suggest that the receptor may be a glycoconjugate [1, 33, 38, 39, 44] but whether it is a glycoprotein or a glycoplipid has remained uncertain.

The role of sialic acid in receptors for many different viruses, including rotaviruses, has been demonstrated using neuraminidase treatment of cells which results in a marked decrease in their susceptibility to virus infection [2, 9, 27, 32, 46]. In vivo and in vitro binding and inhibition of rotavirus by selected sialoglycoproteins has been demonstrated by Yolken et al. [46].

Serum inhibitors may provide information about the structure of the native receptor as they potentially have regions which chemically resemble the important domains of cellular receptors, enabling them to bind to the virus. Serum inhibitors of many viruses including togaviruses [36], reoviruses [35] and myxoviruses [18] have been studied and in some cases, identified [12, 30]. Rotavirus specific inhibitors have been shown to occur in human and calf serum as well as in human milk [6, 40, 45].

In this paper we studied a range of rotavirus serotypes (G1-10) and tested their sensitivity to neutralization by mouse serum. These assays have been followed up with virus overlays of western blots. In addition, reassortants containing the VP4 and the VP7 of different parent strains have used in an attempt to establish whether inhibition is mediated via the VP7 or VP4. The major inhibitor of NCDV rotavirus identified by virus binding assays has been purified and identified.

Materials and methods

Viruses

Human strains used were Wa, ST-3, RV-5, RV-3, B37 and F45, all obtained from J. Albert or R. Bishop of the Royal Children's Hospital, Melbourne, Australia.

Simian rotaviruses SA11, SA11. C1 28 and RRV, and the bovine strains UK and NCDV were kindly supplied by Dr. H. Malherbe, Dr. M. Estes, Dr. T. Flewett, and Dr. H. Greenberg. The porcine strains CRW-8, BEN-307, MDR-13, BMI-1 and TFR-41 and bovine B11 were isolated in our laboratory and kindly provided by Dr. H. Nagesha and Dr. J. Huang.

The turkey rotavirus Ty-1, and the reassortants, 12-1 (RRV × UK), 28-1 (UK × RRV), and 4-1-1 (RV-5 × RRV) were all kindly provided by Dr. H. Greenberg. The reassortant R3/1–2 (UK × SA11)was derived in this laboratory by K. Lucas and 441 (RV-5 × SA11) and SA11 glycosylation variants were obtained from Dr. I. Lazdins. Origins of these strains may be found in [8, 13, 15–17, 20, 26].

All viruses were propagated in MA104 cells in the presence of 0.5 μg/ml trypsin as previously described [16]. Virus was harvested from infected cells by freeze-thawing twice when cytopathic effects were evident. Virus stocks were stored as infected cell lysates at −70 °C.

To obtain purified double-shelled rotavirus particles, cell lysates were prepared as above but in roller bottles (four roller bottles per virus). Four parts tissue culture fluid and one part Arklone (ICI, Australia) were homogenised in a Waring blender for one minute and centrifuged for 10 min at 5 000 rpm to separate the virus containing supernatant. Virus from the aqueous phase was pelleted in a SW28 rotor (Beckman) at 27 000 rpm for 75 min. The virus pellet was resuspended in Tris-saline + calcium (TSC) (50 mM Tris, 20 mM NaCl, 100 mM $CaCl_2$, pH 7.2), loaded onto a glycerol gradient (30–60% v/v) in the same buffer and centrifuged in a SW41 rotor (Beckman) for 75 min at 35 000 rpm. Double and single shelled particles were removed separately from the gradient, resuspended in 7–8 volumes of TSC and centrifuged again in a SW41 rotor for 75 min at 35 000 rpm. Pelleted virus was resuspended in 100 ul TSC and stored at −70 °C.

Serum

Blood was collected from normal BALB/C mice (University of Melbourne, Australia). Serum was obtained by allowing the blood to clot at 37 °C for 30 minutes, pelleting the cells and collecting the serum. The serum was then pooled and heat inactivated at 56 °C for 30 minutes. Storage of the serum was at −20 °C.

Infectivity and neutralisation assays

Infectivity assays to determine virus titres and neutralization assays were conducted according to the methods of Beards et al. [3] with minor modifications [20]. Briefly, serially diluted serum was incubated with a standard inoculum of virus (the dilution of stock which resulted in approximately 400 fluorescent focus forming units/well) for 1 h at 37 °C. The virus-serum mixtures were then transferred to confluent monolayers of MA104 cells grown in tissue culture microtitre plates (Nunc, Kamstrup, Denmark), and incubated for a further hour at 37 °C. The inoculum was then removed, the cells covered with maintenance medium and incubated in a humidified environment with 5% CO_2 for 16 h at 37 °C. Detection of virus infected cells was by indirect immunofluorescence (IF). The inhibitor titre was expressed as the reciprocal of the highest dilution of serum that reduced the number of fluorescent foci by 50% compared to the control value. Titres are the geometric mean of four counts.

ELISA

This was used to detect rotavirus group-specific antibody in preimmune serum. The method was essentially the same as that of Beards et al. [3] describing the detection of rotavirus group-specific antibody. Hyperimmune anti-SA11 serum served as the positive antibody control.

Neuraminidase treatment

Treatment of serum with *Vibrio cholerae* neuraminidase was carried out in certain experiments before sera were used in neutralization assays. One part serum was added to one part receptor destroying enzyme (RDE) (1 000 U/ml) (Whittaker MA Bioproducts, Walkersville, Maryland, U.S.A.) and eight parts calcium magnesium saline, (0.15 M NaCl, 0.25 mM $CaCl_2$, 0.83 mM $MgCl_2$, 0.02 M NaB_4O_7, pH 7.2). The mixture was incubated at 37 °C for 30 min followed by 30 min at 56 °C to inactivate the neuraminidase. Untreated serum went through the same procedure with PBS substituted for RDE.

Treatment of serum with protein A

Protein A treatment of normal serum containing rotavirus inhibitor was carried out using a 50% supension of Sepharose 4B conjugated to Protein A (Protein A-Sepharose CL-4B, Pharmacia, Sweden). 5μl of serum was added to 0.2 ml of Protein A-Sepharose 4B suspension and gently mixed for 15 minutes at room temperature. After a pulse spin at 10, 000 rpm the supernatant was removed and used in neutralisation tests. A mouse monoclonal antibody E1 (IgG 2a) was kindly supplied by Nagesha [26] and used as the positive control.

Virus overlay protein blot assay (VOPBA)

Whole mouse serum diluted 1/150 was separated on a 10% SDS-PAGE gel under reducing conditions. Proteins were transferred to nitrocellulose electrophoretically for 1 h at 100V in a Novex western transfer apparatus as described by Towbin et al. [42]. After blocking for 3 h in 5% skim milk, blots were incubated with 10 ml TSC + 0.1% Tween 20 (TSC-T) containing 3–5 μg/ml purified rotavirus particles for 16 h on a slow shaker at room temperature. The nitrocellulose was then washed 6 times (5 min each) with TSC-T and incubated with hyperimmune rabbit anti-serum (1/500) homologous to the respective virus for 3 h at room temperature. After further washing as described above the blot was incubated with horse-radish peroxidase (HRP)-conjugated sheep anti-rabbit 1g antibody (1/1 000) for 1.5 h at 37 °C. The final wash was done using TSC (without Tween) and the western blot was developed using 30 mg diaminobenzidine and 10mg 4-chloro-1-naphthol diluted in 50 ml TSC until bands became evident. Two nitrocellulose strips treated identically but excluding virus or primary antibody served as controls.

For neuraminidase treatment prior to electrophoresis, serum was incubated for 2 h at 37 °C with 500 m U/ml neuraminidase from either *V. cholerae* or *C. perfringens* (Sigma Chemicals, St. Louis, U.S.A.).

Purification of the mouse serum inhibitor by electro-elution

Whole normal BALB/c mouse serum was boiled for 2 min in an equal volume of Laemmli sample buffer [42] and 60 μl applied to thick (3.00 mm) 8% acrylamide preparative minigels. Gels were stained in 0.05% Coomassie Blue stain (BioRad) in 20% methanol/10% acetic acid, and destained in 40% methanol/10% acetic acid. The desired protein band was carefully excised out of the gels as entire strips and placed in 0.1% SDS in 50 mM NH_4HCO_3 plus 5 mM dithiothreitol (DTT) for 10 min. Gel strips were then placed in dialysis tubing bags containing, and pre-equilibrated with 0.1% SDS in 50 mM NH_4HCO_3. The bags were

sealed and placed in an electrophoresis tank which was also filled with 0.1% SDS in 50 mM NH_4HCO_3 and 50 V applied for 24 h. The "electro-eluate" in the dialysis bag was immediately dispensed into 1.5 ml microtubes and 9 volumes ice-cold methanol added. This was kept at $-20\,^{\circ}C$ overnight and then centrifuged for 5 minutes at 10 000 r.p.m. Protein pellets were dried and incubated for 2 h at $37\,^{\circ}C$ in 0.2% octyl-glucoside (Sigma Chemicals) in tris buffered saline with occasional agitation. The solubilised protein was then pooled, and dialysed for 24 h against 3 changes of 50 mM NH_4HCO_3. Lyophilised aliquots were stored at $4\,^{\circ}C$.

Anti-trypsin activity assay

Anti-trypsin activity was assayed as described in protocols provided by Boehringer Mannheim. Briefly, 50μl aliquots of the trypsin inhibitors (1mg /ml) specified in Table 5, dissolved in 0.1M Tris buffer, pH 8.0 were pre-mixed with 10 μl porcine trypsin (5 μg/ml) (Sigma Chemicals) followed by the addition of 200 μl 1 mM Chromozym-TRY (Boehringer Mannheim), a substrate for trypsin, also dissolved in 0.1 M Tris buffer, pH 8.0. Absorbances were read at 405 nm 30 sec after the Chromozym-TRY was added and inhibitory units/ml calculated as described by Boehringer-Mannheim. This calulation was based on the difference in absorbance measured for reactions without and with trypsin inhibitors present.

Results

Neutralisation of rotavirus by normal mouse serum is not due to antibody

In the course of production of hyperimmune antisera to different strains of group A rotavirus, preinoculation sera obtained from eight week old BALB/c, CBA and BL10 mice were found to have significant serotype specific neutralisation titres (Tables 1 and 2). BALB/c mice were chosen as the model strain for this study. Preimmune serum was tested for rotavirus group-specific antibodies by ELISA using an anti-mouse immunoglobulin conjugate to detect mouse sera that bound rotavirus antigen. All mice tested were free of group specific antibodies against rotavirus. To determine whether the neutralising activity of normal serum was mediated through antibody or non-antibody factors, serum was treated with protein A. A neutralising monoclonal antibody, E1, IgG subclass 2a, to which protein A binds strongly was used as a positive control [26, 41]. Protein A absorption of mab E1 resulted in an eight-fold decrease in neutralisation titres against MDR-13. However test serum treated with protein A did not show a reduction in neutralisation titres against NCDV (serotype 6). This clearly demonstrated that the neutralising activity present in normal mouse serum is mediated through non-antibody factors.

Effects of neuraminidase treatment on serum inhibitor activity

Studies on influenza virus have demonstrated the presence of virus specific alpha inhibitors in normal sera of most species [19], which could be removed by treating the sera with neuraminidase. Similarly, the treatment of normal serum with the neuraminidase from Vibrio cholerae at 1 000 mU/ml was effective in removing rotavirus inhibitory activity. In each case neutralisation titres which ranged between 2 015 and 3 200 dropped to < 100 (Table 2).

Table 1. Neutralisation titres of normal sera from three strains of mice

	Virus mouse strain	Neutralisation titre
NCDV	BALB/c	2 851
	CBA	1 838
	BL10	1 838
RRV	BALB/c	<100
	CBA	<100
	BL10	<100

Table 2. Neutralization titres of normal BALBb/c mouse serum against various rotavirus strains

Strain	G-serotype	Species	Untreated	RDE treated
Wa	1	human	2 540	<100
RV-5	2	human	2 015	<100
RV-3	3	human	2 540	<100
RRV	3	simian	<100	<100
SA11	3	simian	<100	<100
CRW-8	3	porcine	3 200	<100
ST-3	4	human	<100	<100
TFR-41	5	porcine	<100	<100
NCDV	6	bovine	2 851	<100
UK	6	bovine	2 015	<100
Ty-1	7	avian	<100	<100
B37	8	human	<100	<100
F45	9	human	<100	<100
B-11	10	bovine	<100	<100

Mouse serum inhibitors interact directly with rotavirus

To determine whether the serum inhibitor was acting directly upon the virus or if inhibition was a consequence of interaction with the cell monolayer, serum was pre-incubated with the monolayer before the application of rotavirus. As shown in Fig. 1, the susceptibility of MA104 cells was unchanged after incubation with serum, even at the highest concentration of serum used. In contrast, infection by NCDV which had been pre-incubated with serum was inhibited in a dose dependent manner.

Serotype specificity of mouse serum inhibitors

Mouse serum appeared to have no effect on infectivity (titre < 100) of rotavirus serotypes G4, 5, 7, 8, 9, or 10, but had significant neutralisation titres against G serotypes 1, 2 and 6. Variation in sensitivity to inhibition was observed among the G serotype 3 rotavirus strains. Two of these, RRV and SA11 were resistant to inhibition whilst two others, RV-3 and CRW-8, were sensitive (Table 2).

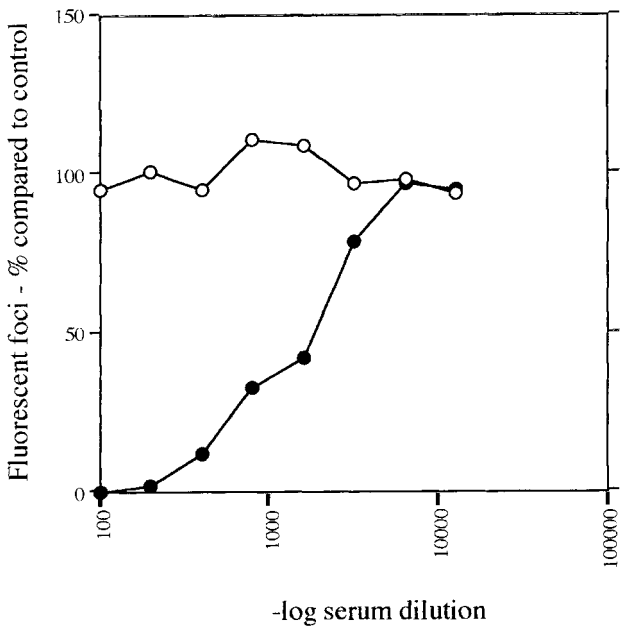

Fig. 1. Comparison of the effects of normal BALB/c mouse serum on NCDV infectivity when incubated with either the virus or the cell monolayer. Serum was serially diluted in 50 μl of virus diluent and was incubated with a standard inoculum of NCDV for 1 h at 37 °C. Monolayers were infected for 1 h with this preparation (●). In parallel, cell monolayers in microtitre trays were incubated with the same final concentrations of serum in virus diluent. This was then removed, the monolayers washed twice with virus diluent and infected with the same final concentration of NCDV (○). The monolayers were incubated for 16 h and stained by immunofluorescence. Infectivity is expressed as the percentage of fluorescent foci of a control infected monolayer

A serotype 3 SA11 variant, V-A10B, which like RV-3, has an additional glycosylation site at amino acid position 238 of VP7, was found to be sensitive to inhibition (titre = 640) while two other variants, V-A10 and Cl.28, which lack glycosylation sites at residue 238 were resistant (titre < 100) (Table 3). Although this suggests glycosylation of VP7 affects reactivity with the serum inhibitor, two other strains, BEN-307 and B11 (G serotypes 3 and 10, respectively) which also have carbohydrate attached at amino acid position 238, were resistant to inhibition by mouse serum. Furthermore, CRW-8 which has no glycosylation site at amino acid 238 is sensitive.

Inhibitor sensitivity correlates with VP7 rather than VP4 in reassortants

The study of reassortants suggested that inhibition is determined by VP7 and not VP4 (Table 4). Each reassortant containing the VP7 of a sensitive strain was sensitive and the reassortant with the VP7 of a resistant strain (12–1) was resistant. Furthermore, reassortants with the VP4 of resistant strains were not always resistant and those with the VP4 of sensitive strains were not always sensitive. For example, reassortant 441 which has the VP7 of the sensitive strain,

Table 3. Effect of glycosylation sites on inhibitor susceptibility of various rotavirus strains and variants

Strain	Glycosylation site	Titre
SA11	69	<100
SA11 C1.28	–	<100
v-SA11-$A10_1$	69, 211	<100
v-SA11-$A10_{11}$	69,238	640
RV-3	69, 238	2 540
BEN 307	69, 238	<100
B11	69, 238	<100
CRW-8	69	3 200

Table 4. Neutralization titres of normal BALB/c mouse serum against various rotavirus strains and reassortants

Virus	Origin of		Titre
	VP4	VP7	
SA11	SA11	SA11	<100
RRV	RRV	RRV	<100
RV-5	RV-5	RV-5	2 015
UK	UK	UK	2 015
RV-3	RV-3	RV-3	2 540
TFR-41	TFR-41	TFR-41	<100
441	SA11	RV-5	2 690
4-1-1	RRV	RV-5	2 425
12-1	UK	RRV	<100
RV-3-TFR	TFR-41	RV-3	2 662
R3/ 1-2	SA11	UK	2 262
28-1	RRV	UK	2 262

RV-5 and the VP4 of the resistant strain SA11 was neutralised to a titre of 1 000. Conversely, the reassortant 12–1, which was resistant to neutralisation, (titre < 100) has the VP7 of SA11 (titre < 100) and the VP4 of UK which is a sensitive strain (titre = 2015).

Identification of virus binding bands

Except in the case of serotype G3 viruses, inhibition appears to be serotype specific rather than host species specific. To determine whether the human strain (Wa) was being neutralised by the same serum component as the animal strains, virus overlay blots of the serum were performed. Wa rotavirus bound to two

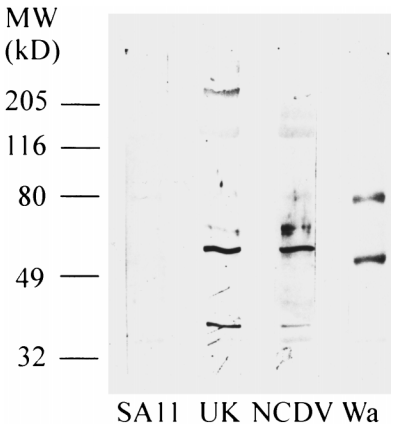

Fig. 2. Rotavirus binding to whole BALB/c mouse serum. Whole mouse serum (1/150) was electrophoresed on a 10% polyacrylamide gel and transferred to nitrocellulose. Cut strips were incubated separately with the specified virus and bound virus was immunodetected as described in Materials and methods

glycoproteins, both of which were different from the one to which NCDV bound, or the two to which UK rotavirus bound. Wa bound to glycoproteins of estimated molecular weight 50 and 80 kDa. NCDV and UK bound to a 55 kDa protein with UK also binding to an additional 36 kDa band (Fig. 2).

Neuraminidase treatment of serum prior to western blotting

The presence of sialic acid is clearly essential for the binding of rotavirus to the serum inhibitors. To determine whether the reduction in neutralisation titre after neuraminidase treatment was reflected by lack of binding on a Western blot, serum was treated with neuraminidase before electrophoresis and assayed as above. Before sampling a range of viruses against neuraminidase treated serum, two different enzymes were compared for their ability to counteract NCDV binding on a blot. As expected the neuraminidase from *V. cholerae* abolished binding. Surprisingly, the neuraminidase from *C. perfringens* was totally ineffective (Fig. 3a).

After treatment of serum with *V. cholerae* neuraminidase the viruses used in Fig. 1 were again tested for their ability to bind on a Western blot. In all cases, binding to the previously identified protein bands was abolished. However, after this treatment the oligosaccharides on some serum proteins were altered so that previously inactive glycoproteins became targets for rotavirus binding (Fig. 3b), in particular to UK, and to SA11 which showed no neutralisation or binding to untreated serum. Though blots are not reliable as a quantitative assay, these bands were generally more faint than those in the untreated serum, possibly explaining the discrepancy between binding on a blot and lack of neutralisation. Alternatively, conformational changes induced by the conditions of the Western blot may facilitate binding which would not occur in the neutralisation assay.

Purification and binding activity of the inhibitor for NCDV

Following the determination on Western blots of the glycoproteins to which rotavirus bound, we sought to purify one of these in order to identify it. The inhibitor

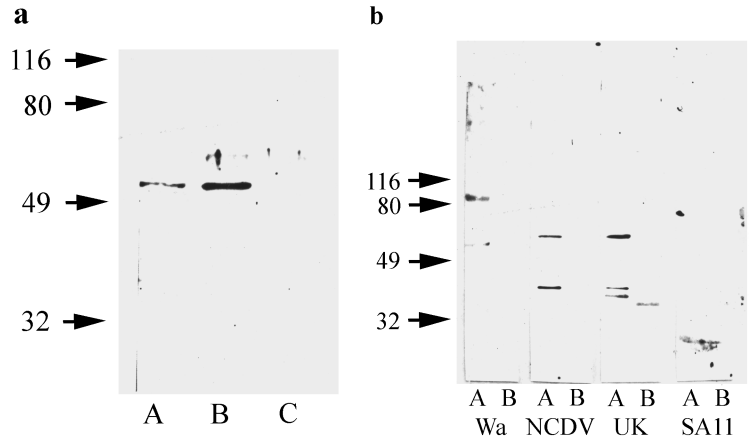

Fig. 3. **a** NCDV binding to neuraminidase treated serum. Whole mouse serum was either *A* mock treated or treated with neuraminidase from *B C. perfringens* or *C V. cholerae.* For *B* and *C* serum was incubated with 500 mU/ml neuraminidase for 2 h at 37 °C followed by boiling for 2 min to inactivate the enzyme. Serum for *A* was treated in the same way but the neuraminidase was omitted. Western blotting and detection of virus binding was as described in Materials and methods. **b** Comparison of viruses; binding after neuraminidase treatment. Prior to electrophoresis serum was either *A* mock treated or *B* treated with *V. cholerae* neuraminidase as described in **a.** Virus binding was detected as described in Materials and methods

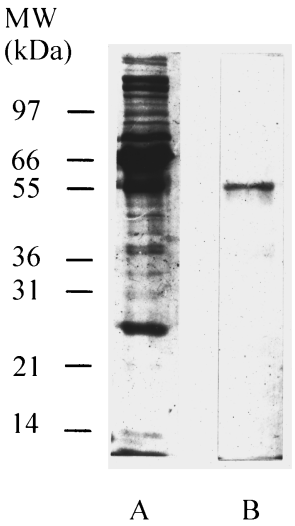

Fig. 4. Purification of the mouse serum inhibitor of NCDV by electro-elution from polyacrylamide gels. Coomassie blue stained polyacrylamide gel showing purification of the serum protein which binds NCDV from whole mouse serum. *A* Whole mouse serum 1/ 150, *B* purified NCDV inhibitor-10 μg

for NCDV was chosen first because it exists as a single glycoprotein. Complete purification of the inhibitor was achieved using the described procedure (Fig. 4). The yield of inhibitor was approximately 10–20 μg/gel onto which 30 μl whole serum was loaded.

To ensure that the purified protein was indeed the inhibitor of NCDV it was tested for its ability to bind rotavirus on a western blot (Fig. 5) and by a neutrali-

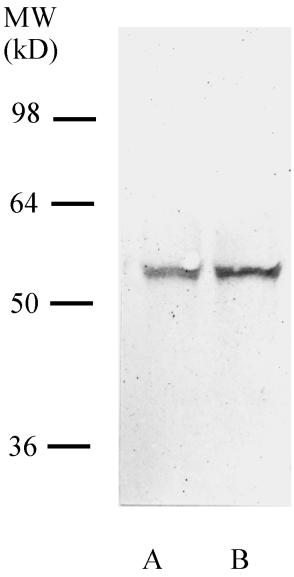

Fig. 5. NCDV binding to purified mouse serum inhibitor. VOPBA analysis of NCDV binding to purified mouse serum inhibitor. *A* Purified mouse serum inhibitor-10 μg, *B* whole mouse serum 1/150

MSI-	Glu-Asp-Val-Gln-Glu-Thr-Asp-Thr-Glu-Gln-Lys-Lys-Gln-Ser-Pro
AAT-	Glu-Asp-Val-Gln-Glu-Thr-Asp-Thr-Ser-Gln-Lys-Asp-Gln-Ser-Pro

Fig. 6. Comparison of N-terminal amino acid sequence of murine alpha-1 anti-trypsin (AAT) and purified mouse serum inhibitor (MSI)

sation assay. Both these methods demonstrated binding by NCDV to the purified inhibitor, indicating the correct protein had been purified and also that it had remained functional with respect to rotavirus binding.

Several other proteins were individually purified and analysed by western blot. NCDV failed to bind to any of these proteins (data not shown) or to serum albumin in the neutralisation assay, indicating that binding was specific. Inhibition of NCDV by purified serum albumin at 15 μg/ml was negligible.

Identification of the inhibitor by N-terminal sequencing and functional assessment

Following automated N-terminal amino acid sequencing the resultant amino acid sequence was found to have 87% (13/15) homology with murine alpha-1 anti-trypsin (AAT) (Fig. 6). The anti-trypsin activity of the purified protein was assessed by its ability to inhibit the digestion of Chromozym-TRY by porcine trypsin. The mouse serum inhibitor showed significant inhibitory activity compared to the co-purified mouse serum albumin and was able to inhibit trypsin digestion at a level comparable to human AAT (HuAAT) (Table 5).

Table 5. Anti-trypsin activity of purified mouse serum inhibitor

Protease inhibitor	Inhibitory activity (Inh. U/ml)
Soybean AT	0.032
Human AAT	0.016
Mouse serum inhibitor	0.017
Mouse serum albumin	0.003

Discussion

The results indicate that normal BALB/c mouse serum efficiently neutralizes several serotypes of rotavirus, including human, bovine and porcine strains. Pre-immune mouse serum with inhibitory activity was found to be free of group specific antibodies against rotavirus by ELISA and protein A treatment of serum had no effect on neutralising activity suggesting the inhibitor is not an antibody. The serum inhibitor was thermostable at 56 °C for 30 min and to boiling for 5 min.

Western blots of normal mouse serum over which virus was incubated revealed that different serum proteins were responsible for the binding of different strains of viruses, particularly among the human and animal strains used in this study. NCDV and UK bound to one common band but UK also bound to an additional, lower molecular weight glycoprotein. It is uncertain whether the latter product is distinct or whether the smaller glycoprotein is a degradation product of the 55 kDa glycoprotein since in some western blots performed using NCDV as the probing virus, this band was also seen.

Wa bound to two proteins, both of which were of different molecular weight from those to which the animal viruses bound (Fig. 2). If carbohydrate is the critical determinant for rotavirus binding, as suggested by numerous authors [33, 44], then these two glycoproteins may share a common carbohydrate. This must, however, differ from any oligosaccharides present on the 55kD glycoprotein. Inhibition of rotavirus infection of MA104 cells only occurred when mouse serum was incubated with virus rather than cells. Hence, it may be suggested that the serum inhibitor exerts its effect by interacting with rotavirus by mechanisms analogous to those by which cell receptors bind to rotaviruses to initiate replication. This has also been proposed for inhibitors of influenza A and B [19] and for a rat serum inhibitor of influenza C [18].

The abolition of inhibitory activity after treating mouse serum with neuraminidase shows that bound sialic acid is essential for effective inhibition. Bastardo and Holmes [2] demonstated that neuraminic acid containing receptors on erythrocytes are involved in haemagglutination by SA11 particles. Similarly, MA104 cell susceptibility to infection by bovine and simian rotaviruses depends on bound sialic acid since hydrolysis of sialic acid moieties using various neuraminidase treatments protects cells from infection [9, 46]. However, as shown by Fukudome et al. [9] human rotaviruses appear to function independently of sialic acid with respect to both haemagglutination and target cell adsorption.

Experiments in this laboratory have confirmed that Wa does not require neuraminidase sensitive sialic acid for the infection of MA104 cells. Yet, the serum inhibitor is able to inhibit Wa infection before neuraminidase treatment of the serum but not after treatment, indicating that neutralization of Wa by this inhibitor is sialic acid dependent.

Thus, Wa binds to a sialylated glycan on the inhibitor which is not present or accessible on the surface of MA104 cells. This gives the impression that the inhibitor is not an analogue of the cellular receptor for Wa. However, attachment to both the inhibitor and the cellular receptor for animal rotaviruses is sialic acid dependent, implying that the inhibitor may well be an analogue of the cellular receptor for animal rotaviruses.

Alternatively, Wa may be able to use another non-sialylated cell surface component that the animal rotaviruses cannot use as a receptor. Or, if the Wa receptor is sialylated, it may be of a variety that is resistant to the neuraminidase of *Vibrio cholerae*, so that while the receptors for animal rotaviruses are destroyed, the human rotavirus receptors are retained.

Thus, although the rationale for studying the serum inhibitor is its potential as an analogue of the cellular receptor, the relationship is complex. Indeed, the ambiguity regarding the nature of the glycoconjugate which acts as the receptor for rotavirus exists because of the various assay systems used to measure rotavirus binding and/or infection. While Yolken et al. [46] showed that several sialoglycoproteins were able to inhibit SA11 infection of MA 104 cells, Rolsma et al. [33] state that mixed brain gangliosides blocked binding of the porcine rotavirus, OSU, to MA104 cells more than any other compounds tested, including a variety of glycoproteins and sialoglycoproteins. Another clear example is the difference in effects of neuraminidase treatment of two different cell lines (MA 104 and Caco-2) on susceptibility to certain rotavirus strains and reassortants [22]. Furthermore, studies which include a rotavirus strain whose species of origin is heterologous to the host cell used may not accurately represent the in vivo situation. ldeally, a murine strain of rotavirus should have been tested for its sensitivity to mouse serum but due to the risk of infecting other laboratory mice, this experiment was not performed. Instead, a number of strains covering a range of serotypes were assayed. These results offer an additional example of the differences in binding patterns between rotavirus strains. Clearly, not every strain of rotavirus is sensitive to inhibition by mouse serum, and different strains are affected by different inhibitors.

Differences in inhibitor suseptibility may be due to differing recognition patterns by the virus of the sialic acid structures on the inhibitor. The HA of Influenza A is capable of recognising and differentiating between specific sialic acid linkages and the oligosaccaride sequences to which they are bound [31, 32]. Specific inhibition of reovirus binding to L-fibroblasts can be mediated only by a2, 3 or a2, 6 linked sialyl-oligosaccharides [27].

Tissue specific sialyltransferase expression has been described by several authors [28]. These reports demonstrate that sialyltransferases act by attaching sialic acids to particular oligosaccarides in unique linkages. The distribution of a

specific sialyltransferase in various tissues may therefore be an important criterion for rotavirus susceptibility of that tissue. This may also have bearing on the species specificity which exists for rotavirus and if so, may also influence the sensitivity of certain rotaviruses to the mouse serum inhibitors.

However, although sialic acid is essential for the binding of various strains of rotavirus to the serum inhibitors, penultimate carbohydrates may be equally important for recognition by those strains and could be the determining factor for resistant strains. Willoughby [44] used a series of oligosaccharides to show that SA11 specifically recognised a trisaccharide. Similarly, both the carbohydrates present on the mouse serum inhibitors and the linkages between them may determine inhibitor activity.

In accordance with the results of the neutralisation assay, binding on a Western blot was prevented after serum was treated with neuraminidase from *V. cholerae*, but *C. perfringens* neuraminidase had little effect. The neuraminidases from *V. cholerae* and *C. perfringens* are reported to have similar substrate specificity [43]; they can cleave either the a2–3, a2–6, or a2–8 ketosidic linkage to the adjacent saccharide unit. Treatment of MA104 cells with these two neuraminidases also affects rotavirus susceptibility of the cells differently (unpubl. data), suggesting a specificity difference between the two enzymes.

It has been proposed that glycoproteins interact with rotaviruses by binding to one of the outer capsid proteins, VP4 or VP7 [10, 14]. Investigation of reassortant susceptibility to neutralization by the mouse serum inhibitor suggested that it is interacting with the VP7 of rotavirus. This conclusion is supported by the phenotypes of two wild type strains, CRW-8 and TFR-41, which show marked differences in the degree of neutralisation by mouse serum. These two strains possess closely related VP4s but have distinct VP7s [15]. Because the inhibitor displayed G serotype specificity, the VP7 amino acid sequences were analysed in an attempt to delineate a region of the VP7 sequence which specified inhibitor susceptibility.

However, between serotypes, no single amino acid change could be detected which neatly differentiated between sensitive and resistant strains. Whether glycosylation of particular amino acids influences inhibitor binding is unclear. Comparison of SA11 variant results with those for wild type SA11 suggest that a glycosylation site at amino acid position 238 enhances binding since the acquisition of this site makes v-SA11-$A10_{II}$ sensitive to inhibition. However, although glycosylation at this site affects the interaction of SA11 with the inhibitor, this is not necessarily the same for other rotavirus strains. Theree rotavirus strains with a glycosylation site at residue 238 were clearly resistant (ST-3, BEN-307 and B-11).

Our results indicate that VP7 is definitely interacting either directly or indirectly with the mouse serum inhibitors. With respect to cell attachment, VP4 is likely to be more important than VP7 as previously outlined in this report. However rotavirus binding and subsequent infection is clearly not a simple process. Mendez et al. [24] suggest that viral entry involves an initial interaction with sialic acid, followed by a second interaction with a specific non-sialylated receptor, both mediated by VP4 [22]. Since neutralisation by the mouse serum

inhibitors is dependent on sialic acid, the results reported here may reflect only the initial attachment step between the virus particle and its target.

It has recently been shown that the parental origin of VP7 may have bearing on the binding specificity of VP4 [25]. Reassortants whose infectivity for MA104 cells depended on the presence of sialic acid on the cell surface were found to possess the same VP4 as variants selected for their ability to enter cells void of sialic acid. Since it has already been established that VP4 is the viral protein which interacts with the cell, the difference can only be attributable to the VP7. In the context of this study, receptor binding would still be mediated by VP4 but the specificity of this interaction would be regulated by the VP7 type associated with the VP4. Given that our findings are based on the study of various reassortants, this evidence could help explain the observed correlation between the VP7 type and sensitivity to inhibition.

The method for purifying the inhibitor was established after much trial and error. Methanol was the only solvent capable of accomodating the amount of SDS required in the electro-elution buffer. Although octyl-glucoside was not the only means of resolubilising the protein following methanol precipitation, it did not inactivate the inhibitory activity and dialysis to remove it was simple. The protein appeared to degrade rapidly in certain solutions, but seemed to be stable at 4 °C once lyophilised.

The isolation and identification of the glycoprotein in serum capable of neutralising at least one and possibly more strains of rotavirus is significant. However, as previously mentioned we believe that the protein is not the critical component of the glycoconjugate to which rotavirus binds. Hence, our future work will focus on the carbohydrates present on MuAAT. As a single molecule, this glycoprotein is an extremely convenient tool for the analysis of the saccharide requirements for rotavirus binding. Of particular interest will be the serum components responsible for human rotavirus neutralisation. It remains to be seen whether the two glycoproteins observed on the western blot are both required for neutralisation of Wa, or whether each can act alone, perhaps by binding to different sites on Wa. Mendez et al. [25] recently illustrated that there are at least two binding epitopes on animal viruses, one of which does not require sialic acid. Similarly, Wa must possess more than one binding site, presumably on its VP4, as binding to MuAAT is sialic acid dependent.

As expected, HuAAT was also able to inhibit trypsin activity although it was ineffective in neutralisation assays of rotavirus (data not shown). HuAAT is known to possess three potential N-glycosylation sites [21]. These positions are preserved in MuAAT [37] but must be occupied by carbohydrate structures sufficiently different to influence rotavirus binding.

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