Sialic acid acts as a receptor for equine rhinitis A virus binding and infection

Rachel A. Stevenson,† Jin-an Huang,‡ Michael J. Studdert and Carol A. Hartley

Centre for Equine Virology, School of Veterinary Science, The University of Melbourne, Parkville, VIC 3010, Australia

Equine rhinitis A virus (ERAV) is a member of the genus Aphthovirus, family Picornaviridae, and causes respiratory disease in horses worldwide. To characterize the putative receptor molecule(s) of the ERAV isolate 393/76 (ERAV.393/76) on the surface of Vero and other cells, an assay was developed to measure the binding of purified biotinylated ERAV.393/76 virions to cells by flow cytometry. Using this assay, the level of binding to different cell types correlated with the relative infectivity of ERAV in each cell type. In particular, equine fetal kidney cells, mouse fibroblast cells, rabbit kidney-13 and Crandell feline kidney cells bound virus at high levels and produced high virus yields (≥ 10^7 TCID_{50} ml^{-1}). Madin–Darby bovine kidney and baby hamster kidney cells showed little or no binding of virus, producing yields of ≤ 10^1–8 TCID_{50} ml^{-1}. Treatment of Vero and other cells with sodium periodate and the metabolic inhibitors tunicamycin, benzyl N-acetyl-α,D-galactosamide, D,L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol and proteases indicated that part of the receptor-binding and entry complex for ERAV.393/76 is on N-linked carbohydrates and that the carbohydrate is likely to be present on a protein rather than a lipid backbone. The effect of carbohydrate-specific lectins and neuraminidases on ERAV.393/76 binding and infection of Vero and other cell types implicated α2,3-linked sialic acid residues on the carbohydrate complex in the binding and infection of ERAV.

INTRODUCTION

Equine rhinitis A virus (ERAV) is an important respiratory pathogen of horses and is classified with foot-and-mouth-disease virus (FMDV), albeit as a separate cluster, in the genus Aphthovirus of the family Picornaviridae. ERAV has been responsible for outbreaks of acute respiratory illness in adult horse populations, although much remains to be learned about the epidemiology and pathogenesis of this virus (Li et al., 1997). Most isolations of ERAV have come from the nasopharynx of horses with acute febrile respiratory disease. ERAV produces viraemia and can be isolated from the blood of acutely ill horses. Horses may carry and shed virus in their urine and faeces for up to 4 weeks post-infection (McCollum & Timoney, 1992; Plummer, 1962). ERAV has a broad host range in vitro and has been reported to infect a number of different cell types, including Vero, rabbit kidney-13 (RK-13), Madin–Darby bovine kidney (MDBK) and equine fetal kidney (EFK) cells (Li et al., 1997; Plummer, 1963; Studdert & Gleeson, 1978). An early study by Lonberg-Holm & Philipson (1974) suggested that ERAV may interact with sialic acid molecules on the surface of cells. However, apart from this study, little is known about the biochemical nature or the cellular function of the receptor(s) involved in ERAV binding to the cell membrane.

While picornaviruses share a range of similar physical properties including structural and sequence similarities, they nevertheless recognize a wide range of different cellular receptors. FMDV is most closely related to ERAV and uses various cell-surface molecules as receptors in vitro. These include the integrins αvβ3, αvβ6, αvβ5, heparan sulphate and Fc receptors (Baranowski et al., 1998; Baxt & Mason, 1995; Berinstein et al., 1995; Jackson et al., 1996, 2000, 2002; Rieder et al., 1996). The major group of human rhinoviruses (HRVs) use intracellular adhesion molecule-1 (ICAM-1) as a cell receptor, while the minor group of HRVs use members of the low-density-lipoprotein receptor family (Greve et al., 1989; Marlovits et al., 1998; Staunton et al., 1989; Tomassini et al., 1989). Many enteroviruses use immunoglobulin superfamily (IgSF) cell-surface molecules such as ICAM-1 as their receptors. Other enteroviruses use cell-surface molecules such as decay-accelerating factor and integrins αvβ3 (vitronectin receptor) and αvβ1 (Roivainen et al., 1994; Xiao et al., 2001). Other IgSF molecules used as receptors by picornaviruses include the cossackievirus–adenovirus
receptor used by many of the coxsackie B viruses and the poliovirus receptor (Rossmann et al., 2002; Newcombe et al., 2003).

In this study, a virus–cell-binding assay was developed, which measured binding of purified biotinylated ERAV isolate 393/76 (ERAV.393/76) to cells using flow cytometry. Using this assay, molecules on several cell lines that bind ERAV and facilitate ERAV infection were characterized using a variety of chemical and enzymic treatments and inhibitors. Biochemical treatments that affected cell binding were further studied using an immunofluorescence infectivity assay (IFA) to determine their effects on cell infection by ERAV.393/76.

METHODS

Virus and cells. Vero cells were grown in minimal essential medium (MEM; Gibco) as described previously (Warner et al., 2001). Other cells used are listed in Table 1. These were grown in MEM medium as above or in MEM containing non-essential amino acids and supplemented with 10% fetal bovine serum (FBS), 50 μg ampicillin ml⁻¹ and 10 mM HEPES, pH 7.4. ERAV.393/76 used in this study has been described previously (Li et al., 1996; Studdert & Gleeson, 1978). Infected cells were maintained at 37°C in MEM or DMEM as above, except that the concentration of FBS was reduced to 0.5%. ERAV.393/76 virions were purified as described previously (Hartley et al., 2001).

ERAV.393/76-binding assay using flow cytometry. Purified ERAV.393/76 virions (50 μg) in 400 μl 50 mM bicarbonate buffer (pH 8.5) were incubated with 2.7 mg EZ-link sulfo-N-hydroxysuccinimide–biotin (Pierce) according to the manufacturer’s instructions. The reaction was stopped by the addition of 40 μl 10× TNE (0.1 M Tris/HCl, pH 8.0, 1.0 M NaCl, 10 mM EDTA). Free biotin was removed by dialysis overnight against PBS with one change of dialysis buffer. Biotinylation of proteins was confirmed by Western blot analysis using horseradish peroxidase-conjugated NeutrAvidin (Pierce) diluted 1:1000. The binding of 0.5 μg biotinylated ERAV.393/76 to cells was detected by flow cytometry as described previously (Warner et al., 2001). Binding to cells was considered positive when the relative linear median fluorescence intensity (RLMFI) value [median fluorescence intensity with biotinylated protein-bound cells/median fluorescence intensity with control cells (no protein)] (Wasserman et al., 1994) was greater than 1.2. To confirm the specificity of the binding assay, 0.05–1.0 μg unlabelled ERAV virions was pre-incubated with cells for 1 h on ice prior to the addition of biotinylated ERAV. In addition, 10 and 100 μg ERAV-specific IgG were prepared from antisera from a rabbit immunized with whole virions, as well as from antisera to recombinant ERAV.393/76 VP1; IgG as a control was similarly prepared from rabbit pre-bleed serum (Warner et al., 2001). These IgG antibodies were pre-incubated with biotinylated ERAV for 1 h on ice prior to the addition of cells.

IFA. Confluent monolayer cultures of Vero cells in 96-well flat-bottomed microtitre plates (Nunc) were infected with approximately 3000 TCID₅₀ ERAV.393/76 (m.o.i. of 0.1) in DMEM for 1 h in 5% CO₂ at 37°C. Following incubation, the cell monolayers were washed twice with DMEM before being incubated in 5% CO₂ at 37°C. After 24 h, the cells were fixed in 90% methanol for 10 min, dried at room temperature and blocked with PBS containing 10 mg BSA ml⁻¹ (BSA10PBS) for 1 h at room temperature prior to the addition of 50 μl primary antibody per well, either rabbit ERAV antiserum or rabbit pre-bleed diluted 1:1000 in PBST (BSA10PBS containing 0.05% Tween 20), and incubation for 1 h. Cells were washed three times with PBST and probed with 50 μl FITC-conjugated swine anti-rabbit antibodies (Dako) at a dilution of 1:40 for 1 h. Plates were washed as above and examined immediately by fluorescence microscopy. The total number of fluorescent cells was counted over four fields at 40× magnification. To examine the inhibition of infectivity, cells were treated or pre-incubated with the proteins, enzymes or lectins of interest (described below), prior to the addition of virus and infection; staining then proceeded as described above. All assays included virus controls (virus and untreated cells only) and cell controls (cells only). Each assay was carried out in triplicate and the results presented as the mean. Results were expressed as a percentage of the virus controls and consisted of the number of fluorescent cells per four non-overlapping fields per well.

Treatment of cells with metabolic inhibitors. Monolayer cell cultures were treated with the glycosylation inhibitors tunicamycin (Sigma) or benzyl N-acetyl-D-galactosamide (benzylGalNAc; Sigma), or with the inhibitor of glycolipid synthesis D,L-threo-1-pheno1-2-decanoylamino-3-morpholino-1-propanol (PDMP; Sigma), essentially as described by Guerrero et al. (2000). Control-cell flasks

Table 1. Binding and infectivity of ERAV.393/76 on various cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Species</th>
<th>Tissue type</th>
<th>Virus binding (RLMFI)</th>
<th>Infectivity titre (log₁₀ TCID₅₀ ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vero</td>
<td>African green monkey</td>
<td>Kidney</td>
<td>5-30</td>
<td>3-80</td>
</tr>
<tr>
<td>BHK-21</td>
<td>Syrian hamster</td>
<td>Kidney</td>
<td>3-92</td>
<td>≤1-80</td>
</tr>
<tr>
<td>COS-7</td>
<td>African green monkey</td>
<td>Kidney</td>
<td>5-63</td>
<td>7-80</td>
</tr>
<tr>
<td>CRFK</td>
<td>Feline</td>
<td>Kidney</td>
<td>12-45</td>
<td>8-05</td>
</tr>
<tr>
<td>EFK</td>
<td>Equine</td>
<td>Kidney (fetal)</td>
<td>23-82</td>
<td>7-80</td>
</tr>
<tr>
<td>EFL</td>
<td>Equine</td>
<td>Lung (fetal)</td>
<td>7-37</td>
<td>6-80</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human</td>
<td>Cervix</td>
<td>10-57</td>
<td>7-05</td>
</tr>
<tr>
<td>McCoy</td>
<td>Mouse</td>
<td>Unknown</td>
<td>20-75</td>
<td>8-30</td>
</tr>
<tr>
<td>MDBK</td>
<td>Bovine</td>
<td>Kidney</td>
<td>3-33</td>
<td>1-80</td>
</tr>
<tr>
<td>MDCK</td>
<td>Canine</td>
<td>Kidney</td>
<td>4-60</td>
<td>5-80</td>
</tr>
<tr>
<td>OFK</td>
<td>Oxine</td>
<td>Kidney (fetal)</td>
<td>7-05</td>
<td>1-80</td>
</tr>
<tr>
<td>RK-13</td>
<td>Rabbit</td>
<td>Kidney</td>
<td>12-51</td>
<td>7-80</td>
</tr>
</tbody>
</table>
were grown and incubated with DMEM without metabolic inhibitor present. After treatment with the respective drug, cells were washed twice with PBS and detached from the flasks as above before being used in the virus-binding assay or being resuspended in 5 ml DMEM and used to seed 96-well trays (100 μl per well) for 4 h for use in the IFA.

**Treatment of cells with sodium periodate (NaIO₄).** Cell suspensions were incubated with 0.011 mM NaIO₄ (AJAX) in PBS for 30 min at 4 °C. Twice the volume of 0.22 % (v/v) glycerol in PBS was added to neutralize unreacted periodate. Cells were washed twice with fluorescence-activated cell sorter (FACS) wash buffer (Warner et al., 2001) before being used in the virus-binding assay. As a control, cells were also mock treated; 0.22 % glycerol in PBS was added to 0.011 mM NaIO₄ in PBS and incubated for 30 min at 4 °C before being added to a cell suspension.

For the IFA, cells in a 96-well tray were treated with NaIO₄ and incubated as above before the reaction was stopped using 0.22 % glycerol. Cells were washed twice with DMEM before being used in the assay.

**Enzyme treatment of cells.** Cell suspensions in 100 μl FACS wash buffer were incubated with 1–50 μg factor X (Sigma), dispase (Difco), trypsin (Gibco) or proteinase K (ICN Biomedicals) or with 1–60 mU Vibrio cholerae (VC) or Clostridium perfringens (CP) neuraminidase (Sigma) for 1 h at 37 °C. Prior to use in the assay, cell suspensions were washed twice in FACS wash buffer. For the IFA, 10 and 20 mU CP neuraminidase in PBS was added to cells in a 96-well tray and the tray was incubated for 1 h at 37 °C. The cells were washed twice with DMEM before being used in the assay.

**Glycosaminoglycan, glycoconjugate and lectin treatment of virus.** Prior to incubation with cell suspensions, biotinylated virions (in 100 μl FACS wash buffer) were incubated with the glycosaminoglycans heparan sulphate, heparin and chondroitin sulphate A, B or C (Sigma) at concentrations of 100 and 1000 μg ml⁻¹, the glycoconjugates sialyllactose (9 mM) and 20 μg colominic acid, fetuin and glycoporphin (Sigma), as well as the lectins Triticum vulgaris agglutinin (wheat germ agglutinin, WGA), Maackia amurensis (MAA) agglutinin, Samucus nigra (SNA) agglutinin, Tetrodonthus parpurus agglutinin (APL) and concanavalin A (ConA) (100 μg ml⁻¹; Sigma).

**RESULTS**

**Detection of binding of ERAV to cells by flow cytometry**

To detect binding of ERAV virions to host cells, a flow cytometry-based binding assay was developed, based on modifications of the methods used by Inghirami et al. (1988) and Ogawa et al. (1998). The assay measured the binding of purified biotinylated ERAV.393/76 to host cells in a dose-dependent manner, where incubation of Vero cells with increasing concentrations of biotinylated virus resulted in a progressive increase in the median fluorescence intensity of the cells (Fig. 1). Multiple preparations of virus yielded consistently similar binding results. Since 0-5 μg biotinylated virus yielded a significant shift in fluorescence, this amount was used for subsequent binding studies, unless otherwise stated. BSA, biotinylated as for purified virus, showed no binding above that of the negative (no protein) control.

![Fig. 1. Dose-dependent binding of biotinylated ERAV.393/76 to Vero cells. The binding of biotinylated ERAV.393/76 to 5 × 10⁵ Vero cells was detected by flow cytometry following staining with streptavidin–FITC diluted 1:100 and compared with a control without virus. The amount of biotinylated ERAV.393/76 added to each tube is shown. The RLMFI values were: control, 1-0; 0-25 μg, 12-27; 0-5 μg, 19-95; 1-0 μg, 42-85; 2-5 μg, 106-4.](http://vir.sgmjournals.org)

To investigate the specificity of binding of ERAV in this assay, unlabelled ERAV.393/76 and ERAV-specific antibodies were used to inhibit the binding of biotin-labelled ERAV to Vero cells. Unlabelled virus inhibited the binding of 0.5 μg biotinylated ERAV.393/76 in a dose-dependent manner, where 0-1, 0-5 and 1 μg unlabelled virus inhibited binding of biotinylated virus by 34, 43 and 100 %, respectively. While this result clearly showed that both labelled and unlabelled virus were competing for the same receptor, it also demonstrated that the effective concentration in the biotinylated virus preparations was likely to be less than 100 %. Random biotinylation of lysine groups on different molecules may have disrupted the binding site on a proportion of the virus particles in these preparations. ERAV.393/76-neutralizing antibodies were also used to investigate further the specificity of the assay. In two separate experiments, 10 μg IgG prepared from ERAV.393/76 antiserum (Warner et al., 2001) completely inhibited binding. It was also shown that 100 μg, but not 10 μg, IgG prepared from GST–VP1 antiserum inhibited ERAV binding to cells. IgG from pre-immune serum from these rabbits did not inhibit virus binding (Warner et al., 2001).

**ERAV.393/76 binds to and infects a range of cell types**

Vero cells were chosen as the cell line for this study as they are routinely utilized in our laboratory for the replication of ERAV isolates, as described by Li et al. (1997). The binding of ERAV.393/76 virions to a range of cell lines was examined...
and the results are shown in Table 1. EFK and mouse fibroblast (McCoy) cells bound ERAV.393/76 to high levels, whereas MDBK and baby hamster kidney (BHK-21) cells showed little or no significant binding. The capacity of each of these cell lines to support replication of ERAV.393/76 was also assessed. As shown in Table 1, the level of ERAV.393/76 binding to these cells generally correlated with the relative infectivity of the virus in the different cell types. In particular, EFK, McCoy, RK-13 and Crandell feline kidney (CRFK) cells bound virus to high levels and produced high virus yields (≥10<sup>7</sup> TCID<sub>50</sub> ml<sup>-1</sup>). MDBK and BHK-21 cells showed little or no binding of virus, producing yields of ≤10<sup>1.8</sup> TCID<sub>50</sub> ml<sup>-1</sup>. Of the cells tested, ovine fetal kidney (OFK) cells showed the least correlation between binding and infectivity, with ERAV.393/76 binding OFK cells to levels comparable with Vero cells, but not supporting replication of virus to titres of >10<sup>1.8</sup> TCID<sub>50</sub> ml<sup>-1</sup>. The inability of ERAV.393/76 to infect these cells, as well as MDBK and BHK-21 cells, was confirmed by immunofluorescence (data not shown).

**Protein is a component of the receptor required for ERAV.393/76 binding**

To assess the role of cell-surface proteins in ERAV binding, cells were treated with a range of proteases. Pre-treatment of Vero cells with the proteases trypsin, proteinase K, dispase or factor X reduced ERAV.393/76 binding in a dose-dependent manner (Fig. 2). At the concentrations used, these proteases did not significantly affect the viability of cells, as assessed by eosin exclusion. Factor X and trypsin in particular, which both cleave peptide bonds at the carboxylic side of arginine, both reduced binding at relatively low concentrations. Over five separate experiments, 2 μg factor X decreased binding by 48–58%, while 50 μg trypsin reduced binding by 52–65%. These results suggested that the ERAV.393/76 receptor on Vero cells contains a protein component.

**An α<sub>2,3</sub>-linked sialylated glycoconjugate is involved in ERAV.393/76 receptor binding**

The role of cell-surface carbohydrate in the binding of ERAV.393/76 was examined by pre-treatment of Vero cells with NaIO<sub>4</sub>. NaIO<sub>4</sub> destroys carbohydrate moieties without altering protein or lipid structures, by oxidation of vicinal hydroxyl groups on sugars to dialdehydes at acidic pH (Martinez-Barragan & Angel, 2001; Woodward et al., 1985). NaIO<sub>4</sub> treatment of Vero cells reduced ERAV.393/76 binding by 70–84% (Fig. 3). These results suggested that carbohydrate is a component of the ERAV receptor. To evaluate the role of N- and O-linked carbohydrates on glycoproteins and glycolipids in ERAV.393/76 binding, Vero cells were treated with metabolic inhibitors of N- and O-linked glycosylation, as well as an inhibitor of glycolipid synthesis. The inhibitors used were tunicamycin, benzylGalNAc and PDMP.

In four separate experiments, it was shown that pre-treatment of Vero cells with tunicamycin reduced ERAV.393/76 binding by 30–45% (Fig. 4a). Pre-treatment of Vero cells with up to 50 μM benzylGalNAc did not reduce binding of ERAV.393/76 to Vero cells. These results indicated that N-linked, but not O-linked, carbohydrates are involved in the binding of ERAV.393/76 to Vero cells. Furthermore, pre-incubation of Vero cells for 3 days with PDMP did not reduce ERAV.393/76 binding significantly. Combined with the results of pre-treatment of Vero cells with proteases, these results indicated that ERAV binds to both a protein and a carbohydrate component on Vero cells. This suggests that ERAV is either binding two separate types of cell-surface molecule or is binding to a single type of molecule, such as a glycoprotein.

To characterize the nature of the carbohydrate component of the putative glycoprotein ERAV.393/76 receptor, Vero cells were pre-treated with neuraminidases from CP and VC, which cleave a broad range of sialic acids. Using 10 mU CP neuraminidase, ERAV.393/76 binding to Vero cells was reduced by 50–65% in five separate experiments and the reduction in binding occurred in a dose-dependent manner. Similarly, 10 mU VC neuraminidase reduced binding by 50–56% in five separate experiments (Fig. 4b). These results indicated that sialic acid is a component of the ERAV.393/76 cell-binding receptor.

---

**Fig. 2.** Effect of protease treatment on ERAV.393/76 binding to Vero cells. Vero cells (5 × 10<sup>5</sup>) were pre-treated with the proteases factor X (FX), trypsin, dispase and proteinase K (PK). Binding of biotinylated ERAV.393/76 to pre-treated cells was detected by flow cytometry. Results presented are from a single experiment and show the best representation of five separate experiments. For each sample, the mean RLMFI value was obtained from duplicate samples and corrected for background binding by subtraction of the mean value obtained for a negative control sample, which was pre-treated but has no virus added. Final results (relative binding) are expressed as a proportion of the RLMFI value obtained for the untreated-virus positive-control samples.
The involvement of sialic acid residues in ERAV.393/76 binding was further investigated using sialic acid-specific lectins. Vero cells were pre-incubated with 100 μg different lectins ml⁻¹ prior to incubation with biotin-labelled virus. In three separate experiments, WGA and MAA lectins, which have an affinity for N-acetylneuraminic acid (NeuAc), N-acetylglucosamine (GlcNAc), (GlcNAc)n and NeuAc2,3-Gal/GalNAc side chains, respectively, inhibited ERAV.393/76 binding by 66–72 % and 60–70 %, respectively (Fig. 5). SNA lectin, which has an affinity for NeuAc2,6-Gal/GalNAc side chains, inhibited binding by 20–30 %. Given the relative binding specificities of these lectins, these results suggested that ERAV shows a strong preference for binding to sialic acids with an α2,3 linkage to the underlying sugar chains. The lectins ConA and APL (with affinities for α-mannose and α-glucose, and NeuAcα2-6Gal/GalNAc side chains, respectively) were also tested. ConA and APL reduced ERAV binding by 70–90 % and 10–13 %, respectively, suggesting that terminal mannose residues of a glycan branched chain or mannose residues within the branched sialylated structure are also involved in ERAV binding. The reduction in binding produced by the lectins WGA, MAA and SNA was dose dependent (data not shown). There was no evidence that these three lectins or ConA caused cell agglutination, as determined by light microscopy and by the forward- and side-scatter profiles of these cells when examined by flow cytometry.

The effect on ERAV.393/76 binding of pre-incubation of virus with the glycoconjugates sialyllactose, colominic acid, fetuin or glycophorin, which contain sialic acids of different types and linkages, was examined. Pre-incubation of 1–0 μg biotinylated ERAV.393/76 with 9 mM sialyllactose (Luo et al., 1997; Zhou et al., 1997) or 20 μg colominic acid, fetuin or glycophorin reduced binding of ERAV.393/76 to Vero cells only slightly, by 5, 17, 15 and 35 %, respectively (data not shown).

**Inhibitors of ERAV.393/76 binding also reduce infectivity**

The attachment of virus to the cell surface may result in specific uptake by the cell as the first step of infection or may result in trafficking to a dead-end pathway where no virus progeny are made. To investigate whether the specific
Inhibition of ERAV binding to Vero cells resulted in a reduction in virus infectivity, compounds known to reduce virus binding to cells were tested for their ability to reduce virus infectivity. Monolayers of Vero cells were pre-treated with ConA, WGA, MAA, SNA, APL, CP neuraminidase, NaIO4 or tunicamycin prior to infection. Virus infection was detected by immunofluorescence after 24 h. All treatments reduced the infectivity of ERAV.393/76 in Vero cells at levels comparable with the reduction in ERAV.393/76 binding (Table 2), with both ConA and NaIO4 treatments reducing infectivity by 80%, and MAA and neuraminidase by 50%. APL had no significant effect on infectivity. PDMP and heparin treatments had no effect on infectivity (Table 2). Lectins, enzymes, glycosaminoglycans and other ligands used in the biochemical characterization of both binding and infectivity of ERAV.393/76 to Vero cells did not modify the measured pH of the medium or the viability of the cells (as assessed by eosin exclusion), which argues for a specific effect of these compounds in reducing the binding to and infection of Vero cells by ERAV.393/76.

**Evaluation of receptor properties in other cell lines**

ERAV infection of cells and animals from a wide range of hosts is consistent with the finding that ERAV can bind to sialic acids, since these carbohydrates are ubiquitous on the surface of many different types of cells. To examine whether ERAV.393/76 uses sialic acid as a receptor on cell types other than Vero cells, and in light of the observation that ERAV.393/76 was able to bind and infect several other cell lines to higher levels than Vero cells, sialic acid-specific inhibitors were investigated for their effects on the binding of ERAV.393/76 to four other cell types [Madin–Darby canine kidney (MDCK), EFK, RK-13 and CRFK] found to bind and support replication of ERAV.393/76 to high levels (Fig. 6). As shown for Vero cells, treatment of each of the cell types with NaIO4 resulted in a reduction in binding of 50–80%, also suggesting that ERAV binds to a carbohydrate on the surface of these cells. Similarly, treatment of cells with neuraminidase resulted in a significant reduction in ERAV.393/76 binding to all cells except EFK cells, where only a minor reduction in binding was seen. WGA and MAA lectins significantly reduced ERAV binding to Vero and MDCK cells; although they also had some effect on reducing binding to EFK and RK-13 cells, the reduction was comparatively small. MAA had no effect on the binding of ERAV to CRFK cells. Taken together, these results indicate that ERAV may not utilize exactly the same cell-binding receptor on different cell types. However, because most of the treatments that had an effect on ERAV.393/76 binding to Vero cells also had some effect on the other cell types used, the receptor used by ERAV on these cell types must share common features.

### Table 2. Effect of binding inhibitors on the infectivity of ERAV.393/76 in Vero cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inhibitor concentration</th>
<th>Infectivity (% of virus control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaIO4</td>
<td>0.011 M</td>
<td>24.6*</td>
</tr>
<tr>
<td>Mock</td>
<td>0.011 M</td>
<td>100</td>
</tr>
<tr>
<td>Neuraminidase (VC)</td>
<td>100 mU ml⁻¹</td>
<td>59.6</td>
</tr>
<tr>
<td>Neuraminidase (CP)</td>
<td>200 mU ml⁻¹</td>
<td>54.8</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>2 μg ml⁻¹</td>
<td>67</td>
</tr>
<tr>
<td>PDMP</td>
<td>25 μg ml⁻¹</td>
<td>147</td>
</tr>
<tr>
<td>Tunicamycin + PDMP</td>
<td>2 + 25 μg ml⁻¹</td>
<td>55</td>
</tr>
<tr>
<td>ConA</td>
<td>100 μg ml⁻¹</td>
<td>16.6</td>
</tr>
<tr>
<td>MAA</td>
<td>100 μg ml⁻¹</td>
<td>54.5</td>
</tr>
<tr>
<td>WGA</td>
<td>100 μg ml⁻¹</td>
<td>74.3</td>
</tr>
<tr>
<td>SNA</td>
<td>100 μg ml⁻¹</td>
<td>73.3</td>
</tr>
<tr>
<td>APL</td>
<td>100 μg ml⁻¹</td>
<td>92.5</td>
</tr>
<tr>
<td>Heparin</td>
<td>100 μg ml⁻¹</td>
<td>108</td>
</tr>
</tbody>
</table>

*Expressed as a percentage of the amount of infectivity observed in the mock-treated control cells.
DISCUSSION

The findings presented in this study provide evidence that sialic acid acts as a receptor for the binding and infection of ERAV. Results of initial general characterization studies indicated that carbohydrate is a component of the ERAV393/76 cell-binding receptor complex. Furthermore, we found that neuraminidase inhibited ERAV393/76 binding to Vero cells by up to 60%, indicating that ERAV393/76 was interacting with a sialic acid-containing cell-surface receptor. A reduction in binding of ERAV393/76 to Vero cells following protease treatment of cells was detected by flow cytometry. For each sample, the mean RLMFI value was obtained from duplicate samples and corrected for background binding by subtraction of the mean value obtained for a negative control sample, which had no virus added. Final results (relative binding) are expressed as a proportion of the RLMFI value obtained for the untreated-virus positive-control samples.

Sialic acid moieties are acylated derivatives of neuraminic acid (5-amino-3,5-dideoxy-D-glycero-D-galactononulosonic acid) and are frequently found on glycoproteins or glycolipids, usually at the free terminus of short, often branched oligosaccharide chains (Burness, 1981; Alberts et al., 1989). Several picornaviruses use sialic acid for binding to the surfaces of susceptible cells, including bovine enterovirus, HRV-87 and persistent strains of Theiler’s murine encephalomyelitis virus (Zhou et al., 1997; Evans & Almond, 1998; Alexander & Dimmock, 2002; Jnaoui et al., 2002). An early study by Lonberg-Holm & Philipson (1974) also indicated that ERAV may interact with cell-surface receptors containing sialic acid, since pre-treatment of HeLa cells with neuraminidase prevented the binding of radiolabelled ERAV but not the binding of HRVs and poliovirus.

Sialic acid molecules gain diversity in their structure by variation in the type of sialic acid (usually NeuAc or GlcNAc) or by the type of linkage of the sialic acid to the underlying sugar chain. The ability of both WGA and MAA, but not SNA, lectins to inhibit ERAV binding significantly suggests that ERAV has a preference for binding to sialic acid with an α2,3 linkage to the underlying sugar residues. Both CP and VC neuraminidases have a preference for NeuAc sialic acids over GlcNAc sialic acids. Taken together, these results suggest that ERAV393/76 shows a preference for binding to NeuAc sialic acid with an α2,3 linkage to the underlying sugar residues. ConA, a lectin that binds to terminal mannose residues of a glycan branched chain or mannose residues within the branched sialylated structure (Varki et al., 1999), also strongly inhibited binding of ERAV to Vero cells. This suggests that either mannose or glucose residues also participate directly in receptor binding, or that ConA sterically inhibits binding of virus to cells by binding mannose residues proximal to sialic acid side chains on the glycan.

It has been suggested that non-enveloped viruses require a specific protein entry receptor for internalization into the cell (Reddi & Lipton, 2002). While the concentration of tunicamycin used in this study may have affected the glycosylation of both proteins and lipids (Ogawa et al., 1998), the inability of PDMP to reduce binding to cells suggests that ERAV is binding to sialic acid on the surface of N-linked glycoproteins. The reduction in binding demonstrated after protease treatment of cells supports this notion.

The ubiquitous nature of sialic acid on the surface of cells is consistent with the ability of ERAV to bind to and infect a range of cell types from a range of host species. However, the effects on ERAV393/76 binding when a range of cells was treated with WGA, MAA and SNA lectins and neuraminidase were variable. This might occur as a result of variation in the types of sialic acid presented on the different cells, where stronger binding of ERAV to a particular sialic acid may be more difficult to inhibit with these lectins. Allaway & Burness (1987) demonstrated that treatment of the sialylated glycoprotein glycoporphin with neuraminidase prevented it binding to encephalomyocarditis virus–Sepharose columns, indicating a requirement for sialic acid for receptor activity. However, sialylglycoconjugates such as fetuin, which binds to specific sialic acids, were unable to bind the virus. This indicates that the presence of sialic acid alone on a molecule is insufficient to result in binding and that other features of the molecule must be important. Glycoporphin was the strongest inhibitor of binding, reducing binding by 35%, and this glycoconjugate is known to contain a mixture of sialic acid molecules. While the substrate specificity of neuraminidases and lectins may cleave and bind to a relatively broad range of sialic acid
moieties, the sialic acid molecules presented on individual glycoconjugates may be more limited and as such may not contain exactly the right conformation required for ERAV binding.

The process resulting in infection of a cell by a virus is complex. Binding of virus to more than one surface molecule has been demonstrated for several viruses. In a recent biochemical study of rotavirus receptors, it was shown that at least three cell-surface molecules were involved in the early steps of the interaction of rotaviruses with MA104 cells (Guerrero et al., 2000). For other viruses such as adenovirus 9 (Ad9) and Ad19, it is known that binding to a cell does not necessarily lead to infection (Arnberg et al., 2000). Interestingly, inhibitors that prevented ERAV.393/76 binding to sialylated molecules on the surface of Vero cells reduced the infection of Vero cells by ERAV.393/76 to levels comparable with their effect on ERAV.393/76 binding. However, none of the treatments resulted in complete inhibition of binding or infection, which suggests that another molecule(s) may be involved in the binding and infection of Vero cells by ERAV. The existence of a second receptor for ERAV, in addition to sialic acid, could provide an alternative explanation for the reduction in binding of ERAV after protease treatment of cells.

The results presented in this study provide preliminary data on the types of receptor molecules that may be involved in ERAV.393/76 binding and infection of cells, and indicate that ERAV may utilize more than one receptor molecule. Further studies are required to elucidate the nature and involvement of these receptor molecules in more detail.

ACKNOWLEDGEMENTS

We thank Nino Ficorilli and Cynthia Brown for excellent technical assistance. We also thank Dr Elizabeth Washington for assistance with the flow cytometry analyses. This work was supported in part by Racing Victoria and a Special Virology Fund. R. A. S. was the recipient of a University of Melbourne Research Scholarship.

REFERENCES


