Attachment of bovine parvovirus to sialic acids on bovine cell membranes

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Although it has previously been shown that bovine parvovirus (BPV) attaches to the sialated glycoprotein glycophorin A on erythrocytes, the nature of virus-binding moieties on mammalian nucleated cells is less clear. Buffalo lung fibroblasts (Bu), primary bovine embryonic kidney cells, Madin–Darby bovine kidney cells and bovine embryonic trachea (EBTr) cells were assessed for molecules capable of binding BPV. Competition studies were carried out on both erythrocyte and nucleated cell targets using a variety of sialated compounds and sialic acid-negative compounds. Glycophorin A was found to inhibit BPV binding, while mucin exhibited low-level inhibition. These two sialated compounds also blocked attachment of BPV-modified microsphere carriers to the Bu cell membrane. Influenza A virus was used as a sialic acid competitor and interfered with BPV attachment to erythrocytes and replication in Bu cells. Significantly, the enzyme sialidase removed BPV-binding sites from Bu and EBTr cells. The binding sites could be reconstituted on sialidase-treated cells by the enzymes α-2,3-O-sialyltransferase and α-2,3-N-sialyltransferase. These results indicated that BPV can attach to sialic acid on cell membranes and that the sialylglycoproteins available for virus attachment appear to contain both N- and O-linked carbohydrate moieties, but that not all members of the sialic acid family can bind BPV. Moreover, there may be other moieties that can bind BPV, which may act as either primary or secondary receptors.

INTRODUCTION

Autonomous parvoviruses replicate independently of the helper viruses required by the dependent parvoviruses, but require host cells in S phase for complete replication. Early steps in the replication cycle, such as attachment and penetration, occur independently of S phase but await cell permissiveness established in S phase for genome replication (Leary & Storz, 1980; Pritchard et al., 1981). Bovine parvovirus (BPV), a pathogen of cattle causing gastroenteritis and mild respiratory symptoms (Spahn et al., 1966), is an autonomous, non-enveloped, icosahedral, single-stranded DNA virus. Its genome consists of two non-identical inverted terminal repeats that flank two ORFs, one of which encodes two non-structural proteins while the other encodes the three structural proteins found in the capsid (Chen et al., 1986, 1988; Johnson & Hoggan, 1973).

The erythrocyte receptors for some other parvoviruses have been characterized. Feline panleukopenia virus (Goto, 1975; Mochizuki et al., 1978), canine parvovirus (Barbis et al., 1992) and minute virus of mice (Tullis et al., 1993) use a sialoglycoprotein as their receptor, and human parvovirus B19 binds to a carbohydrate other than sialic acid on the human blood group P antigen (Brown et al., 1993). Studies on the receptors for parvoviruses on nucleated cells that initiate virus replication show that adeno-associated virus type 4 (AAV-4) (Kaludov et al., 2001) and AAV-5 (Walters et al., 2001) bind to sialic acid residues, while AAV-2 binds to heparan sulfate (Summerford & Samulski, 1998; Qiu et al., 2000) as well as human fibroblast growth factor 1 (Qing et al., 1999) and αVβ5 integrin (Summerford et al., 1999). Aleutian mink disease virus (AMDV) is reported to bind to a 67 kDa protein named the AMDV binding protein (Fox & Bloom, 1999). In addition, canine parvovirus binds to a number of sialylglycoproteins on the surface of nucleated cells (Barbis et al., 1992).

Early BPV–cell interactions are incompletely understood. It has been reported that BPV attaches to glycophorin A, but does not bind to asialoglycophorin A (Thacker & Johnson, 1998). Glycophorin A is a transmembrane sialoglycoprotein found in erythrocyte membranes (Tomita et al., 1978). The glycophorin A monomer is a type 1 membrane glycoprotein composed of 131 aa with three domains: a hydrophilic cytosolic domain, a hydrophobic transmembrane domain and the amino-terminal hydrophilic external domain. Glycophorin A naturally exists as a homodimer (Furthmayr & Marchesi, 1976) with a total molecular mass...
of about 78.5 kDa. There is extensive O-linked oligosaccharide glycosylation on the external domain (Challou et al., 1994; Tomita et al., 1978), suggesting that this virus may bind to O-linked sialic acids. This interaction mediates the haemagglutination (HA) reaction, but glycoporin A, because of its limited cellular distribution, does not mediate attachment of this virus to nucleated bovine cells required for virus replication. This study was undertaken to begin to identify the virus-binding sites used by BPV on permisive, nucleated bovine cells.

METHODS

Viruses and cells. The BPV used in this study was the original strain of BPV obtained from F. R. Abinanti (National Institutes of Health, Bethesda, MD, USA; Abinanti & Warfield, 1961). It was grown and purified as described previously (Johnson & Hoggan, 1973). Briefly, the virus was grown in primary bovine embryonic kidney (BEK) cells, buffalo lung fibroblasts (Bu; ATCC IMR-31) or bovine embryonic trachea cells (EBTr; ATCC NBL-4). For BEK cells, the culture medium was composed of 50 % Eagle's No. 2 medium and 50 % medium 199 (Sigma Aldrich) containing 0-03 % glutamine, 50 µg penicillin ml\(^{-1}\), 50 µg streptomycin ml\(^{-1}\), 100 µg neomycin ml\(^{-1}\) and 10 % heated fetal bovine serum (FBS). For Bu and EBTr cells, Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich) containing 0-11 % sodium bicarbonate, 10 mM HEPES buffer, 50 µg gentamicin ml\(^{-1}\) and 5 % cosmetic calf serum (HyClone) was used. For virus stocks and for purified virus, cell sheets were infected at 60-70 % confluence to ensure the presence of mitotic cells. Before infection, the cells were washed with serum-free medium to minimize any contaminating anti-BPV antibody introduced into the cultures by the bovine serum. Virus was allowed to adsorb for 6 h, then serum was added to a concentration of 2 %. Incubation was carried out at 37 °C. When complete cytopathic effects were achieved (4–6 days post-infection), the cultures were either freeze-thawed three times and aliquoted for virus stocks, or processed for purification. Purification was done by ultracentrifugation, as described previously (Johnson & Hoggan, 1973). The virus in infected cells and medium was pelleted by ultracentrifugation, the pellets were suspended and the virus was further purified by banding three times in isopycnic CsCl gradients.

Some inhibition tests were carried out in Madin–Darby bovine kidney (MDBK) cells (ATCC CCL-22).

Influenza A virus, strain PR8 (ATCC VR-95), was grown in Madin–Darby canine kidney cells (ATCC CCL-34) in DMEM with 10 % FBS.

Human type O erythrocytes were collected in Alsever’s solution and stored at 4 °C. Before use, they were washed three times in PBS, then diluted to a concentration of 0-5 % (v/v).

Inhibitors. The compounds used in the competition assays to test for inhibition of HA and inhibition of infection were purified glycoporin A, asialo glycoporin A, fetuin, asialofetuin, mucin, N-acetyl neuraminic acid (from Escherichia coli), N-acetyl neuraminic acid (synthetic) and heparan sulfate (all from Sigma Aldrich).

HA assays. These were performed in 96-well U-bottomed plates (Corning no. 25881-96). Serial twofold dilutions of BPV were made in 50 µl gelatin/BSA buffer (0-005 % gelatin and 0-1 % BSA in PBS, pH 7.0) and 50 µl of a 0-5 % erythrocyte suspension was added. The plates were incubated at 4 °C overnight. Positive results were scored as agglutinated cell sheets compared with non-agglutinated red cell buttons. HA titres were expressed as the minimum virus concentration required to haemagglutinate the erythrocyte suspension completely.

In the HA inhibition tests, the test components in each well were gelatin/BSA buffer, 4 HA units of BPV antigen and inhibitor (as part of a dilution series) or control wells without inhibitor. This mixture was incubated for 30 min at room temperature to allow binding of virus to the inhibitor, then washed red blood cells were added, mixed and incubated overnight at 4 °C. Erythrocyte cell controls were also included. The amount of HA was scored on a scale of 0 to 4-.

Infectivity assays. These tests were carried out in either 24-well tissue culture plates or flat-sided tissue culture tubes (Nunc). The virus stock used in the tests had an infectious titre of 10\(^7\) TCID\(_{50}\) ml\(^{-1}\) and was diluted to contain a countable number of focus-forming units (ffu). The test components per well or per tube consisted of a dilution of inhibitor in serum-free medium (DMEM), or no inhibitor in control wells or tubes, and an amount of virus corresponding to 60–130 ffu. The virus concentration varied from assay to assay, but was always a consistent amount within each experiment. This mixture, with a maximum inhibitor concentration of 250 µg ml\(^{-1}\), was incubated for 30 min at room temperature to allow binding of the virus to inhibitor. Host cells in serum-free medium were added drop-wise in suspension and the contents of the culture mixed and incubated for 1 h at room temperature with periodic agitation to allow virus attachment to cell receptors. DMEM containing 2 % FBS was added and the cultures were incubated at 36 °C for 2 days. The monolayers were fixed in formaldehyde/ethanol/acetic acid (FAA) fixative (Luker et al., 1991) and stained with guinea pig anti-BPV antibody using an immunoperoxidase method described previously (Luker et al., 1991), with the following modification: the chromogen consisted of 20 mg 4-chloro-1-naphthol in 100 ml of a solution composed of 2 ml DMSO, 10 ml 95% ethanol, 0-1 ml 30 % hydrogen peroxide and 87-9 ml distilled water.

In an alternative culture format, cells were processed as monolayer rather than being added to cultures in suspension. In this type of culture, the cells were grown to 70 % confluency and washed with serum-free medium. Virus added for an adsorption time (described in Results), fed with growth medium and incubated, then stained with immunoperoxidase to reveal the foci of infected cells.

In cultures tested for influenza virus infection of BPV attachment, monolayers of Bu cells were grown to approximately 70 % confluency in flat-sided tubes. The monolayers were washed in serum-free medium (DMEM) and inoculated with varying amounts of influenza A virus, strain PR8, followed by inoculation of BPV. Virus attachment was allowed to proceed for 2 h at 36 °C. At this time the cultures were again washed with serum-free medium to remove unattached virus, then the cultures were renewed with medium containing 10 % FBS. They were incubated at 36 °C for 48 h, fixed in FAA fixative and stained with immunoperoxidase as described above to identify infected cells.

Immunofluorescence. The nucleated bovine cells used for virus infection were tested for the presence of glycoporin A in their cell membranes. Control erythrocytes were tested simultaneously as positive controls. Cells growing on round coverslips in shell vials were washed with PBS, fixed in cold acetone, then stained with anti-glycoporin A (MAB050; Chemicon), followed by FITC-labelled goat anti-mouse immunoglobulin (Chemicon) with Evans blue counterstain. Red cell smears and red cell ghosts were tested simultaneously as controls. Observations of fluorescence were carried out using a Nikon fluorescence microscope.

Latex particles. Sulfate latex particles (Interfacial Dynamics) of 1-9 µm diameter were loaded with isopycnic CsCl gradient-purified BPV antigen following a procedure provided by the manufacturer.
The BPV-microsphere carrier complex was allowed to attach to cells in monolayers. Unattached spheres were removed by washing with PBS, and the formation of complexes was assessed by observation using bright-field microscopy. Attachment of clusters of particles demonstrated attachment of virus to cell surfaces. Mixing soluble inhibitors, with a maximum inhibitor concentration of 600 \( \mu \)g ml\(^{-1}\), with the latex carriers prevented virus attachment in cases where the inhibitor coated the virus antigen.

**Dot blots.** Twenty micrograms of each potential binder along with control dots were dotted on to nitrocellulose membranes, allowed to dry and the membranes were blocked using a blotto blocker (Thacker & Johnson, 1998). The membranes were then washed three times with sample buffer (3 g powdered milk in 100 ml PBS). Following washing, the membranes were incubated with BPV antigen (8 HA units ml\(^{-1}\)), washed and the dots visualized by treatment with anti-BPV antibody, immunoperoxidase conjugate and the 4-chloronaphthol chromogen as detailed above. In tests assessing the possible blocking of BPV binding by influenza virus, the dots were pre-incubated with influenza virus before the BPV antigen was applied.

**Binding of radiolabelled virus.** BPV grown in BEK cells, labelled with \(^{14}\)C-labelled mixed amino acids and purified on isopycnic CsCl gradients, was mixed with human type O erythrocytes to assess binding. Suspensions of erythrocytes washed in PBS were reconstituted to a concentration of 1% (v/v) red cells in PBS. Aliquots of red cells were mixed with varying amounts of \(^{14}\)C-labelled purified virus and incubated at room temperature for 2 h to allow attachment to proceed. The cells in the mixture were separated by low-speed centrifugation and the cell pellets and supernatants were independently mixed in Ecoum scintillation fluid (ICN Pharmaceuticals). The bound and unbound radioactivity was counted in a Beckman liquid scintillation counter.

In inhibition experiments, influenza A virus (strain PR8) was added to red cell suspensions and incubated for 1 h at room temperature followed by overnight incubation at 4°C. The radiolabelled BPV was added and processed as described above, with scintillation counting distinguishing between bound and unbound virus.

**Stripping of BPV receptors by sialidase.** Neuraminidase (sialidase) preparations from Vibrio cholerae and from Clostridium perfringens (Roche) were dissolved in serum-free medium and used to treat either Bu or EBT\(\text{r}\) cells. The cells were digested with these enzymes either in suspension as unattached cells or as cells attached to culture tubes as monolayers. Both of the enzymes removed the BPV receptors with comparable efficiency. Therefore, the neuraminidase from V. cholerae was used for stripping of the BPV receptors in most of the studies reported here. Enzyme activity was confirmed by showing dose-responsive sialic acid cleavage. This enzyme exhibits broad substrate specificity, hydrolysing terminal N- or O-acetylated acrylic acids in oligosaccharides, polysaccharides, mucopolysaccharides, glycoproteins and glycolipids that show \(\alpha-2,3\), \(\alpha-2,6\) or \(\alpha-2,8\) bonds. It is free from proteases according to the manufacturer's data sheet.

**Reconstitution of BPV receptors by sialyltransferases.** Duplicate monolayer cultures of Bu cells were grown to 70% confluency in flat-sided culture tubes. Stripping of the BPV receptors was carried out with 5 mU V. cholerae neuraminidase ml\(^{-1}\) for 1 h at 37°C. The cultures were washed in serum-free medium (DMEM) and each was treated with 0-7 mg cytidine 5'-monophospho-N-acetylmuramic acid (the neuraminic acid donor) and either \(\alpha-2,3\)-O-sialyltransferase or \(\alpha-2,3\)-N-sialyltransferase (EMD Biosciences) at varying concentrations for 1-5 h at 37°C. The cultures were washed with serum-free medium and inoculated with 1x10^6 infectious units of virus in 0.1 ml. Following an adsorption time of 2 h at 37°C, the cultures were washed with serum-free medium to remove unattached virus, then fed with growth medium containing 10% cosmic calf serum and incubated at 37°C for 48 h. At this stage, the cells were fixed in FAA fixative and stained with the peroxidase stain as described above. Foci of infected cells were scored by examination by light microscopy and were counted either as f.f.u. per culture or as mean number of f.f.u. per microscopic field. Each culture was examined for infected cells independently by two readers and mean results were calculated.

## RESULTS

### Inhibition of BPV-mediated HA

Human erythrocytes with their primarily O-linked sialated residues on glycophorin A were used as a model to determine whether other forms of sialated substances would interfere with the binding of BPV to the red cell substrate. This was done to help assess whether the virus–sialic acid interaction was narrowly restricted to a limited form of sialic acid or whether virus binding would occur with a broad range of sialic acid types. Since sialic acids consist of a family of acylated N- and O-linked carbohydrates with various \(\alpha\)- and \(\beta\)-glycosidic bonds, it is not assumed that all members of the sialic acid family will bind the virus. Mucin, fetuin and N-acetylmuramic acid from two sources, as well as asialofetuin and heparan sulfate, were tested as potential inhibitors of virus binding to red cells. Heparan sulfate was tested because it is a receptor for some other paroviruses such as AAV-2 and also other unrelated viruses such as herpesviruses.

Preparations of virus particles purified on isopycnic CsCl gradients were tested for HA activity. The amount of virus was standardized to contain 4 HA units in inhibition tests. Virus was mixed with various amounts of inhibitor ranging from 10 \(\mu\)g to 1 ng and the binding reaction was allowed to proceed. Unbound virus was measured by HA. The results shown in Fig. 1 demonstrated inhibition of HA with glycoporin A but not with any of the other substances tested. The effect of removal of the sialic acid residues from glycoporin A on its ability to bind virus was tested using asialoglycoporin A (Fig. 1). Asialo glycoporin A did not bind the virus; therefore, the sialic acid residues on glycoporin A are essential for virus binding, and in their absence glycoporin A protein is insufficient for virus attachment.

### Attachment of virus to nucleated cells

Glycoporin A is found on the surface of some erythroid cells and some cells from malignancies of various organs but is not found on the surface of all nucleated somatic cells. If BPV attaches to sialic acids on non-erythrocytes, the receptors may be sialated glycoproteins other than glycoporin A. To determine whether BPV would attach to such sialic acids, a series of experiments was performed. Assays detecting infectious virus were employed in which BEK cells, MDBK cells, Bu fibroblasts and EBT\(\text{r}\) fibroblasts were used as target cells. These cells were screened by immunofluorescence for the presence of glycoporin using a monoclonal...
antibody that reacts with the glycophorin protein. No glycophorin was detectable in their membranes (data not shown) but glycophorin could be detected in control erythrocytes by this method. If sialic acids are used as BPV receptors, it might be expected that exposure of the virus to sialated compounds would result in virus binding to these substances and prevention of virus attachment to cells, thus inhibiting virus replication in the target cells. Because glycophorin A is known to bind the virus, it was expected that exposure to this substance would thus prevent infection. This would occur irrespective of the nature of the cell receptor. However, other sialated compounds not active in inhibiting HA could inhibit infection if other members of the sialic acid family were used as cell receptors. Moreover, if specific sialidases removed the receptor(s), it would provide evidence that this virus can use cellular sialic acids to initiate infection.

The same group of inhibitors described above was used to test for inhibition of virus replication in BEK (Fig. 2) and MDBK cells. Virus was mixed with various amounts of inhibitors ranging from 50 µg to 5 pg and the binding reaction allowed to proceed. Free infectious virus detected as f.f.u. in cultures in 24-well tissue culture plates. As expected, glycophorin A inhibited infection in both cell types (data for MDBK cells not shown). Asialoglycophorin A did not inhibit infection in BEK cells in control cultures. Therefore, the virus was capable of binding to the sialic acids on glycophorin A and binding could be measured by loss of infectivity. Some inhibition of infectivity was observed with other sialated compounds.
mucin (Fig. 2). The low-level activity seen with mucin suggested that the virus may bind to sialic acids other than glycophorin A. Notably, $N$-acetylneuraminic acid did not bind to the virus, as was the case in the HA models, suggesting that this virus recognizes sialic acids that differ from those recognized by influenza virus or that this virus ligand–receptor interaction requires the glycosidic linkage to a penultimate carbohydrate. Multiple repeat experiments yielded data similar to those shown in Fig. 2.

To assess binding of virus to the sialeted compounds in a more direct manner, dot blots were performed. The test compounds dotted on to a nitrocellulose membrane were exposed to virus and virus attachment was revealed using anti-BPV antibody binding to the virus–receptor complex and immunoperoxidase staining. The results (Fig. 3) showed virus binding strongly to glycophorin A and weakly to mucin. These results confirmed the observations that BPV binds to sialic acids other than glycophorin A but not to free $N$-acetylneuraminic acid.

**Confirmation of BPV binding to mucin and glycophorin A using microsphere carriers**

Virus capsids and capsid proteins isolated on isopycnic gradients were bound to the surface of latex microspheres. These particles are large enough to be seen by light microscopy. Exposure of the microspheres to permissive cells in monolayer culture permitted binding of the carrier complex on to the surface of susceptible cells, which could be identified by microscopy as cells having clusters of particles bound to their cell membrane. When the carriers were exposed to receptors in soluble form prior to cell attachment, inhibition of attachment occurred if the soluble receptor bound to the virus on the latex particles. The results shown in Fig. 4 show the attachment of virus–microsphere complexes to BEK cells. In the absence of inhibitor (control) or in the presence of $N$-acetylneuraminic acid, binding of the carriers occurred, but in the presence of mucin or glycophorin A, binding was inhibited, confirming the attachment of the virus to these two substances.

**Removal of BPV receptors from cells and reconstitution of receptors**

Bu and EBTr cells in monolayer cultures were digested with 5 mU sialidase and immediately infected with virus. Treated cells were compared with control untreated cells for virus replication by identifying numbers of infected cells (f.f.u.). Infection was performed immediately after removal of the enzyme to minimize the number of receptors reappearing

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**Fig. 3.** Dot blot showing binding of BPV to 20 μg dots of sialated, non-sialated and asialated compounds. There was high level binding of virus to glycophorin A, weak binding to mucin (scored by eye on a scale of 0 to 4+) and no detectable binding to the other compounds, including asialoglycophorin A.

**Fig. 4.** Sialylglycoprotein interference with BPV attachment to the membranes of BEK cells. Microspheres loaded with BPV capsid proteins attached to BEK cells growing in monolayers. By light microscopy, positive cells could be seen as cells covered with clusters of microspheres. Negative cells have no microspheres or only a few random, single spheres. Pre-treatment of the BPV–microsphere complexes with glycophorin A (Glyc A) or mucin blocked cluster formation, while pre-treatment with $N$-acetylneuraminic acid ($N$-AcNAcid) did not.
on the cell membranes as a result of cellular repair of the membranes. The results in Fig. 5(a) (Bu cells) and (b) (EBTr cells) showed that the number of infected cells was reduced by approximately 50% following sialidase treatment, indicating that the treatment was effective in stripping away the sialic acid receptors from enough cells to reduce the available number of host cells to approximately half that of the control cells. In a subsequent experiment, functional receptors used for BPV attachment were reconstituted on sialidase-treated Bu cells. Cell monolayers were treated as before with sialidase, reducing the number of permissive cells by approximately 50% (Table 1). Such cultures were exposed to sialytransferases and an N-acetylneuraminic acid donor to determine whether receptors capable of initiating BPV infection could be reconstructed on the cell membrane. \(\alpha\)-2,3-\(N\)-sialytransferase, at concentrations of 10 and 25 mU, re-formed much of the functional receptor activity, as did \(\alpha\)-2,3-\(O\)-sialytransferase at the same levels of activity. The re-formed receptors did not reach 100% of the original activity, so in another experiment a higher amount of enzyme was used (50 mU). Again, the reduction of infected cells was approximately 50% (Table 2). With higher enzyme activity, the permissive cells in the cultures available for virus replication almost reached pre-digestion levels. The observation that increasing levels of sialytransferases increased the available receptors suggests that the reconstituted receptors resulted from the sialytransferase treatment in a dose-dependent manner and not from natural cell-mediated resialation. The data also suggest that more than one form of sialic acid can serve as the BPV-binding site.

Other experiments were performed in which the cells were exposed to sialidase with the cells in suspension rather than in monolayers. These treated cells were then infected and allowed to attach and grow as monolayers. It was thought that cells in a spherical conformation might be more efficiently treated by sialidase than cells flattened in monolayer culture where the receptors on the bottom side of the cells might be unavailable for enzymic digestion. The results showed that cells treated for receptor removal in suspension were extremely vulnerable to cell death by the stripping method and that most cells then became unavailable for virus replication.

**Table 1. Reconstitution of BPV receptors on Bu cells following neuraminidase removal**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Enzyme activity (mU)</th>
<th>No. of f.f.u. per culture*</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>128</td>
<td>100</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>5</td>
<td>71</td>
<td>56</td>
</tr>
<tr>
<td>(\alpha)-2,3-(N)-Sialytransferase</td>
<td>10</td>
<td>124</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>109</td>
<td>85</td>
</tr>
<tr>
<td>(\alpha)-2,3-(O)-Sialytransferase</td>
<td>10</td>
<td>98</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>117</td>
<td>91</td>
</tr>
</tbody>
</table>

*Mean of the number of f.f.u. calculated from four replicate cultures.

**Table 2. Resialation of BPV receptors on Bu cells with high levels of sialytransferase**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Enzyme activity (mU)</th>
<th>No. of f.f.u. per culture*</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>990</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>5</td>
<td>548</td>
<td>55</td>
</tr>
<tr>
<td>(\alpha)-2,3-(N)-Sialytransferase</td>
<td>50</td>
<td>984</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>927</td>
<td>94</td>
</tr>
</tbody>
</table>

*Mean of the number of f.f.u. calculated from two replicate cultures.
Assessment of inhibition of BPV attachment by influenza virus

Since the sialidase substrate specificity was broad and both N- and O-linked reconstituted receptors served as attachment sites for BPV, it is believed that this virus can attach to several sialic acids in the broader sialic acid family. However, none of the previous data indicated that N-acetylneuraminic acid serves as a BPV receptor as it does for influenza virus. If these two viruses use common receptors, influenza virus might be found to be an inhibitor of BPV attachment. Dots consisting of 20 μg glycophorin A were applied to nitrocellulose membranes. To determine whether pre-exposure of the dot to influenza virus would block attachment of BPV, dots were treated with influenza virus at a concentration of 64 HA units, followed by BPV, and visualized by peroxidase staining. The positive control was treatment with BPV without influenza virus and the negative control had neither influenza virus nor BPV to assess non-specific binding. Influenza virus did not perceptibly block attachment of BPV to glycophorin A, indicating that the sialic acids on glycophorin A to which BPV attaches may not be within the binding range of influenza A virus (Fig. 6).

In other experiments, influenza virus appeared to block attachment of BPV to human type O erythrocytes (data not shown). Pre-exposure of erythrocytes to influenza virus inhibited attachment of 14C-labelled BPV in a dose-dependent manner. Moreover, in infectious assays carried out in Bu cells, influenza virus inhibited the appearance of BPV infectious centres, again in a dose-dependent manner. The results of the tests involving binding to red blood cells and the infectivity tests on Bu cells where influenza virus interfered with BPV attachment were somewhat unexpected since soluble N-acetylneuraminic acid failed to bind BPV. It is possible that the two viruses share some common receptors in the much larger constellation of sialic acids on cell membranes. Alternatively, it may be that influenza virus simply sterically hinders or covers some BPV receptors on erythrocytes and nucleated Bu cells, making them unavailable for BPV attachment.

DISCUSSION

The results of this study have demonstrated for the first time that BPV is capable of attaching to sialic acids on nucleated bovine cells and initiating the virus replication cycle. Moreover, the binding sites consisted of both O- and N-linked sialic acid forms. Furthermore, the strong binder, glycophorin A, was a major receptor for BPV on the membranes of erythrocytes.

N-Acyl derivatives of neuraminic acid, generically called sialic acids, usually exist as the terminal sugar on complex polysaccharides. When linked to proteins through serine or threonine residues they comprise the O-linked sialic acids, and when linked through asparagine they comprise the N-linked sialic acids. The sialidase used in these studies removed the terminal neuraminic acid residue from the complex. The enzyme α,2,3-O-sialyltransferase attaches the neuraminic acid residue as the terminal sugar on O-linked side chains and the enzyme α,2,3-N-sialyltransferase carries out this process on N-linked side chains. The specificity of these two enzymes for forming O- and N-linkages, respectively, was shown previously by Kaludov et al. (2001). The fact that sialidase removed the BPV receptor from Bu and EBTr cells showed the BPV receptor to be composed of sialic acid and that binding sites for the virus are available on buffalo and cattle cells. Since both sialyltransferases restored BPV attachment to cells, this suggested that both O- and N-linked sialic acids can function as BPV-binding sites.

The glycophorin homodimer is a major component of the erythrocyte membrane. It is also found on nucleoid erythroid precursor cells. It attaches to the band 4.1 protein in the cytoskeletal structure through the glycophorin cytosolic domain, penetrates the plasma membrane with an α-helical transmembrane domain and its major glycosylated domain is extracytoplasmically oriented. The exoplasmic domain has 16 carbohydrate residues attached to it, 15 of which are O-linked through serine or threonine, with one longer one being N-linked through asparagine. This study did not determine whether both the O-linked and the one N-linked residue bound BPV. The fact that glycophorin A is probably not the receptor on nucleated cells in culture is consistent with the observation that immunofluorescence using a glycophorin-specific monoclonal antibody did not reveal glycophorin on the bovine cell lines but did show glycophorin A on the erythrocyte membrane.
Mucins are a class of glycoprotein composed of acid mucopolysaccharides, with the acidic group being either a carboxyl or a sulfuric group. They contain heteropolysaccharides with two types of alternating monosaccharide units, one of which is the acidified moiety. The second moiety, the sialic acid residue, is contained in a disaccharide with the additional acetyl group present as O-acetyl. This study did not include identification of the BPV-binding target on mucin, but it was observed that binding to this substance, as measured in the competition tests, was much weaker than binding to glycoporin A and cell receptors. Mucin was found to compete with cell receptors for BPV in cultures infected with this virus but was not observed to inhibit HA, presumably manifestations of the difference in sensitivity between the HA and the infectivity tests. A higher concentration of mucin in the microsphere-binding tests compared with the infectivity tests exhibited greater activity.

It has been shown that glycoporin A cross-reacting epitopes are present on non-erythroid tissues, as well as on erythroid cells. These structures are found, in addition to erythroleukaemia cells, on melanomas and carcinomas of the breast, cervix, larynx, nasopharynx and colon, as well as normal human fibroblasts and mammary cells (Barsoum et al., 1984). Moreover, antigenic cross-reactions have been shown between human and bovine glycoporin A, but not between human glycoporin A and that from chickens, guinea pigs, horses, rabbits, sheep or swine (Shan et al., 1998). Human type O erythrocytes were used in the studies reported here, but elsewhere it has been reported that guinea pig erythrocytes are also haemagglutinated by BPV, whereas bovine, chicken or rhesus erythrocytes are not haemagglutinated (Abinanti & Warfield, 1961). In fact, BPV-infected cells can be detected in haemadsorption tests using human and guinea pig red blood cells. Why humans and guinea pigs are apparently not within the BPV host range is unknown, but it may be because the red cell HA receptors are different from the somatic cell receptors required for infection.

It has been reported that AAV-5, another parvovirus, also binds to 2,3-N-linked sialic acid, while AAV-4 binds to 2,3-O-linked sialic acid (Walters et al., 2001; Kaludov et al., 2001). Interestingly, our studies showed that BPV bound to both of these sialic acid forms. These results are a significant first step in identifying the BPV cell receptors. Furthermore, influenza viruses isolated from humans attach to 2,6-linked sialic acids (Rogers & Paulson, 1983; Rogers et al., 1983). The fact that influenza virus blocked BPV binding may indicate that BPV can use 2,3-linked as well as 2,6-linked sialic acids. However, it should be noted that influenza A virus neuraminidase is reported to cleave both 2,3-linked and 2,6-linked sialic acids (Franca de Barros et al., 2003). This activity (receptor removal) may account for the inhibition of BPV binding mediated by influenza A virus strain PR8 observed in the current study. Further investigation will be required to clarify this point. Moreover, since free neuraminic acid did not block BPV attachment, this suggests that neuraminic acid must be linked to a neighbouring residue for binding to occur. These parameters are under further investigation.

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REFERENCES


BPV attaches to bovine cell membrane sialic acids