Binding of bovine parvovirus to erythrocyte membrane sialylglycoproteins

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Bovine parvovirus (BPV), an autonomous parvovirus, haemagglutinates human type O erythrocytes and infects certain bovine cells in culture. Little is known about the receptor to which it attaches, either on nucleated host cells or on erythrocytes. Haemagglutination assays and radiolabelled virus-binding tests measuring the effects of trypsin, chymotrypsin, neuraminidase, phospholipase C and sodium periodate on attachment of BPV to receptors indicated that BPV interacted with N-acetyl-neuraminic acid-containing (sialyl) glycoproteins. SDS–polyacrylamide gel separation of erythrocyte ghost proteins and virus overlay protein-binding revealed BPV binding to glycophorin A. Confirmation testing showed BPV binding to purified glycophorin A on dot blots and on gels containing membrane glycophorin A and purified glycophorin A. Further, in competition assays, purified glycophorin A completely inhibited the BPV haemagglutination reaction. The results of this study indicate that BPV binds to sialated membrane glycoproteins, one of which is the major erythrocyte membrane glycoprotein, glycophorin A.

Introduction

Bovine parvovirus (BPV), a pathogen of cattle, was originally isolated from the gastrointestinal tract of calves with diarrhoea by Abinanti & Warfield (1961). BPV was shown to cause mild diarrhoea in calves inoculated per os, and when inoculated intranasally it caused diarrhoea and mild respiratory symptoms (Spahn et al., 1966). Further, BPV was isolated from cattle in outbreaks of infection (Storz et al., 1972) and serosurveys indicated a high incidence of infection in some herds (Abinanti & Warfield, 1961; Sandals et al., 1995; Storz et al., 1972).

BPV, as a member of the family Paroviridae, is a small (22 nm), non-enveloped, icosahedral, single-stranded DNA virus. It is an autonomously replicating parvovirus as opposed to the adeno-associated viruses, which are largely defective viruses that require co-infection with a virus helper. Five BPV virus-encoded proteins have been reported, three of which are capsid proteins (Johnson & Hoggan, 1973; Lederman et al., 1983) and the other two are the nonstructural proteins NS-1 (molecular forms of $M_r$ 75 000 and $M_r$ 83 000) (Lederman et al., 1987) and the phosphorylated protein NP-1 ($M_r$ 28 000) (Lederman et al., 1984). The capsid proteins are VP1 ($M_r$ 80 000), VP2 ($M_r$ 72 000) and VP3 ($M_r$ 62 000). VP3 comprises more than 75% of the virion protein, while VP1 and VP2 make up the remainder of the capsid protein in approximately equimolar ratios (Johnson & Hoggan, 1973).

Parvovirus DNA is linear, single-stranded and composed of about 5000 nucleotides. It contains terminal palindromic sequences and, in several parvoviruses, both plus and minus DNA copies are encapsidated during the assembly process. The entire nucleotide sequence of BPV DNA has been reported (Chen et al., 1986). It is a sequence of 5491 nucleotides containing non-identical palindromic termini. There are three open reading frames (ORFs) on the plus strand. The right ORF encodes the three structural proteins and the left and centre ORFs encode the nonstructural proteins (Chen et al., 1986).

Glycophorin A is a major transmembrane glycoprotein found in erythrocyte membranes (Tomita et al., 1978). The glycophorin A monomer is composed of 131 amino acids found in three domains: a hydrophilic cytosolic domain, a hydrophobic transmembrane domain and the amino-terminal hydrophilic external domain. There is extensive O-linked oligosaccharide glycosylation on the external domain (Challou et al., 1994; Tomita et al., 1978). Glycophorin A naturally exists as a homodimer (Furthmayr & Marchesi, 1976). Thus, its total molecular mass is about 78 500 daltons.

Although many molecular biological characteristics of BPV are known, much of the biology of the virus–cell interaction...
remains obscure. One of the characteristics of several of the parvoviruses is their ability to agglutinate erythrocytes. BPV has haemagglutination (HA) activity for both human type O and guinea-pig erythrocytes (Bates et al., 1972). The erythrocyte receptors for four paroviruses have been characterized. Feline panleukopenia virus (FPV) (Goto, 1975; Mochizuki et al., 1978), canine parvovirus (CPV) (Barbis et al., 1992) and minute virus of mice (Tullis et al., 1993) use a sialic acid-containing glycoprotein as their receptor. The parovirus B19 binds to a carbohydrate other than sialic acid on the human blood group P antigen (Brown et al., 1993). However, little information has been reported on the interaction of the BPV capsid or its proteins with either erythrocyte receptors or host cell receptors.

This paper reports that BPV binds to sialated erythrocyte membrane glycoproteins and attaches to the major membrane glycoprotein, glycoporphin A.

**Methods**

**Virus and cells.** BPV was grown and purified as previously described by Johnson & Hoggan (1973). Briefly, the original strain of BPV, obtained from F.R. Abinanti (National Institutes of Health, Bethesda, MD, USA), was grown in either primary bovine embryonic kidney cells or buffalo lung fibroblasts (ATCC, Bu, IMR-31). The culture medium was composed of half Eagle’s no. 2 and half medium 199 containing 0·03% glutamine, 50 µg/ml each of penicillin and streptomycin and 100 µg/ml neomycin and 10% heated foetal bovine serum. Cell sheets were infected at 60–70% confluence to assure the presence of mitotic cells. Before infection, the cells were washed with serum-free medium, then virus was allowed to adsorb for 0·5 h. Following adsorption, serum was added to a final concentration of 2%. Incubation was at 37 °C. When complete cytopathic effects were achieved (4–6 days post-infection), the virus particles and antigen were harvested by centrifugation as previously described (Johnson & Hoggan, 1973). The virus in infected cells and the medium was pelleted by ultracentrifugation, the pelleted virus was further purified by three-times banding in isopycnic CsCl gradients.

Influenza type A virus, strain PR8 (ATCC VR-95), was grown in Madin–Darby canine kidney cells (ATCC CCL 34). The medium used was Dulbecco’s modified Eagle’s medium, containing 10% foetal bovine serum, 0·11% sodium bicarbonate, 10 mM HEPES buffer and 50 µg/ml gentamicin.

Human type O erythrocytes were collected in Alsever’s solution and stored at 4 °C. Before use they were washed three times in PBS (8 g/l NaCl, 0·2 g/l KCl, 1·15 g/l Na2HPO4, 0·2 g/l KH2PO4 at pH 7·4), then diluted to a concentration of 0·5% (v/v).

**Haemagglutination assay.** The HA assay was performed in 96-well U-bottom 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). Then 50 µl of 0·5% erythrocyte suspension was added. The plates were incubated at 4 °C overnight. Positive results were scored as agglutinated cell sheets compared to non-agglutinated red cell buttons. HA titres were expressed as the minimum virus concentration required to haemagglutinate completely the erythrocyte suspension.

**Enzyme treatment of erythrocytes.** Washed human type O erythrocytes were treated for 1 h at 37 °C with one of the following enzymes obtained from Sigma: 0·05 and 0·1 U/ml neuraminidase (type V from *Clostridium perfringens*); 0·1 g/ml trypsin (1:250, from porcine pancreas); and 0·5 and 0·1 mg/ml chymotrypsin (type 1-S from bovine pancreas). Following incubation, the erythrocytes were washed three times in PBS and used in HA assays as described above. Enzymatic treatment with 5 × 10⁻³ and 5 × 10⁻⁴ units of phospholipase C (type 1 from *Clostridium perfringens*) was performed at 37 °C for 15 min, then the erythrocytes were washed and used in the HA assay. At these enzyme concentrations, treatment of the erythrocytes did not cause lysis, so the red cells remained intact for use in HA assays.

**Treatment of erythrocytes with periodate.** Sodium periodate, at final concentrations of between 0·1 and 1 mM, was added to washed type O erythrocytes and incubated for 15 min at 37 °C. An equal volume of 1% (v/v) glycerol was added to neutralize any unreacted periodate. The erythrocytes were then used in the HA assay.

**14C-labelled virus-binding assay.** Washed human type O erythrocytes were diluted to 1% (v/v) and treated with one of the following enzymes: 0·05 and 0·1 U/ml neuraminidase, 0·1 and 0·05 g/ml trypsin, and 0·5 and 0·1 mg/ml chymotrypsin for 1 h at 37 °C. The erythrocytes were then washed three times with PBS. After the final wash, the erythrocyte pellets were diluted to 1% (v/v). BPV labelled with 14C-labelled mixed amino acids and purified on CsCl isopycnic gradients was added and allowed to adsorb onto the erythrocytes for 1 h at room temperature. The erythrocyte suspension was centrifuged to separate the erythrocytes from the supernate. The supernate and the erythrocytes were each added separately to 10 ml Scinti Verse I scintillation fluid and the radioactivity counted in a Beckman liquid scintillation counter.

**SDS–PAGE analysis of proteins.** Erythrocyte ghosts were prepared by the method of Dodge et al. (1963) as outlined by Fairbanks et al. (1971). Briefly, the erythrocytes were washed three times in PBS (5 mM sodium phosphate pH 8·0), then lysed in lysis buffer (salt-free 5 mM sodium phosphate pH 8·0). The ghosts were then washed with lysis buffer until they were white or slightly pink. Ghost proteins and purified glycoporhin A (obtained from Sigma) were separated by electrophoresis on 10% SDS–polyacrylamide gels in a Bio-Rad Mini–Protein II apparatus, according to the manufacturer’s directions. After electrophoresis, the proteins were either electrophoretically transferred to nitrocellulose membranes or stained. The proteins in the gels were stained with either Coomassie brilliant blue or periodic acid–Schiff (PAS) reagent (Matthieu & Quarles, 1973; Segrest & Jackson, 1972) to visualize the glycoproteins.

**Western blot and dot blot tests.** The Western blot tests were Western blot-like tests using the virus as a probe to attach to nitrocellulose membrane-bound receptor molecules. This technique has been referred to as a virus overlay protein-binding procedure (Nieper & Müller, 1996). Nitrocellulose membranes, to which either the separated membrane components or purified glycoporhin A were transferred, were blocked by incubating the strips in blotto blocker (0·1 g sodium azide, 5·0 g powdered milk, 10 µl antifoam A in 100 ml PBS) for 1 h. All incubation steps for the Western blot were performed at room temperature. The blocker was aspirated and nitrocellulose was washed three times with sample buffer (3·0 g powdered milk per 100 ml PBS pH 7·0). Purified BPV diluted 1:20 in distilled water (final concentration of 8 HA units/ml) was added and the strips agitated for 1 h. The BPV solution was then washed and the membranes washed three times with sample buffer. Guinea-pig antiserum prepared against the native BPV whole capsid antigen (diluted 1:1000, final antibody concentration of 200 complement fixing units/ml) was then added. This mixture was incubated with agitation for 1 h. The nitrocellulose membranes were washed three times with sample buffer. Sample buffer was added and agitated for 4 min. The sample buffer was removed and horseradish

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peroxidase–protein A enzyme conjugate (diluted 1:500 in PBS containing 1% BSA) was added and incubated with agitation for 45 min. The membranes were then washed four times with sample buffer. Deionized distilled water was added and agitated for 4 min. After removing the water, the chromogen (0.56 mM 4-chloro-1-naphthol, containing 0.003% hydrogen peroxide as the enzyme substrate) was added. The plate containing the nitrocellulose membrane strips was agitated several times and the colour allowed to develop for 10 min. The chromogen was removed and the strips were flooded with deionized distilled water and allowed to stand for a minimum of 5 min. The nitrocellulose strips were then dried and stored in the dark.

In the dot blot experiments, purified glycophorin A was dotted onto nitrocellulose membranes in a Bio-Rad dot blot template, allowed to dry, then blocked, probed with virus, antiviral antibody, enzyme conjugate and substrate as described above.

Results

Effect of enzymatic digestion of erythrocyte surface on virus binding

To begin to characterize the chemical nature of the erythrocyte HA receptor to which BPV attaches, human type O erythrocytes were treated with trypsin, chymotrypsin, phospholipase C, neuraminidase and sodium periodate. These modified erythrocytes were tested for their virus-binding activity in HA assays. All the enzymes used for the membrane digestion experiments were tested at the experimental concentrations against known substrates as positive controls to verify enzymatic activity (data not shown). Treatment of the erythrocytes with trypsin and chymotrypsin reduced the ability of the cells to bind BPV (Table 1). In these experimental conditions, enzyme digestion did not lyse the erythrocytes. These results indicated that an erythrocyte membrane-associated protein was involved in attachment of BPV.

Table 1. Effect of enzymatic digestion and periodate treatment of human type O erythrocytes on HA and the attachment of BPV to the erythrocyte surface

<table>
<thead>
<tr>
<th>Enzyme (concentration)</th>
<th>Haemagglutination* (Relative%)</th>
<th>Bound radiolabelled virus (%)†</th>
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<tbody>
<tr>
<td>Trypsin (mg/ml)</td>
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<tr>
<td>0</td>
<td>100</td>
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<tr>
<td>50</td>
<td>ND</td>
<td>64</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>59</td>
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<tr>
<td>Chymotrypsin (mg/ml)</td>
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<td></td>
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<tr>
<td>0</td>
<td>100</td>
<td>100</td>
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<tr>
<td>0.1</td>
<td>50</td>
<td>94</td>
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<tr>
<td>0.5</td>
<td>25</td>
<td>81</td>
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<tr>
<td>Phospholipase C (µU/ml)</td>
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<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
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<tr>
<td>50</td>
<td>100</td>
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<td>500</td>
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<td>Periodate (mM)</td>
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<td>ND</td>
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<tr>
<td>0.1</td>
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<td>ND</td>
</tr>
<tr>
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<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>ND</td>
</tr>
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<td>Neuraminidase (U/ml)</td>
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<td>20</td>
</tr>
<tr>
<td>0.10</td>
<td>&lt; 6</td>
<td>26</td>
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</tbody>
</table>

* Enzyme-treated erythrocytes (0.5% suspension) were haemagglutinated with twofold dilutions of virus. Percentage equals end-point titre compared to control HA titre.
† BPV labelled with 14C-labelled amino acids was bound to control and enzyme-treated erythrocytes. Percentage equals fraction of radioactive counts attached to the treated erythrocytes compared to control erythrocytes.
ND, Not done.

Identification of the virus receptor by SDS–PAGE

To determine the identity of the membrane glycosylated protein that binds BPV, erythrocyte ghosts were isolated and the membrane components were separated in several lanes on SDS–polyacrylamide gels. Gels were divided into thirds. One-third of the lanes was used for protein transfer onto nitrocellulose for use in the Western blot assays. One-third of the lanes was stained with Coomassie blue to visualize the proteins, and the remainder of the gel was stained with the PAS stain to visualize the glycoproteins. These results are shown in Fig. 1. The membrane components were separated in lanes 1 and 3. Lane 1 was stained with Coomassie blue and lane 3 was stained with PAS. Lane 2 contains the molecular mass reference markers. There was a major PAS-staining band at Mr 78,500. This band is referred to as PAS band 1 (Fairbanks et al., 1971), which was renamed glycophorin A by Furthmayr et al. (1975). The Western blot of this separation demonstrating BPV binding to the glycophorin A band is shown in lane 4.
Separation of erythrocyte membrane proteins by SDS–PAGE and binding of BPV to the glycophorin A components. Erythrocyte ghost components were separated by SDS–PAGE and stained with Coomassie brilliant blue (lane 1), a PAS stain (lane 3), or transblotted to nitrocellulose and stained for receptor using BPV as the probe (lane 4). Protein standards were run for comparison (lane 2): BSA, 66 kDa; chicken egg albumin (OA), 45 kDa; and trypsin inhibitor from soybean (TIS), 20 kDa.

Binding of BPV to purified glycophorin A. In a dot blot format, 12.5 µg purified glycophorin A per dot was blotted onto two nitrocellulose strips and probed for BPV receptor activity with virus (b) or without virus in a control reaction (a). Another control dot (c) contained no glycophorin A but was probed with virus. For the dots to appear, both glycophorin A and virus were required and, as shown in dot (b), BPV attached to glycophorin A.

To confirm the ability of BPV to bind to glycophorin A, dot blot experiments were performed. Purified glycophorin A (12.5 µg) was blotted onto each of two spots on separate nitrocellulose strips. After blocking, one strip was treated with a virus suspension and the other strip was mock-treated as a control. A dot containing no glycophorin A, but probed with virus, was included as an additional control. Following virus exposure, the strips were treated with antiviral antibody, enzyme conjugate and enzyme substrate as described in Methods. The results shown in Fig. 2 demonstrate specific virus binding to the glycophorin A dot, confirming the finding that BPV can bind to the glycophorin A molecule. In another dot blot experiment, a series of tenfold dilutions of purified glycophorin A was dotted onto a nitrocellulose membrane and probed as above. The dots contained 10 µg to 0.1 ng glycophorin A in the concentration series. Positive dots were obtained in the dots containing 10 µg, 1 µg, 100 ng and 10 ng (results not shown). The dots containing 1 ng and 0.1 ng were negative. The stain intensity of the positive dots diminished as the glycophorin A concentration decreased in the series. This result again confirmed the binding reaction of BPV to glycophorin A and, furthermore, it was in a manner dependent upon the glycophorin A concentration.

Binding of BPV on erythrocyte membrane glycophorin A compared to binding on purified glycophorin A

Erythrocyte ghost proteins were separated by SDS–PAGE in parallel with purified glycophorin A (Fig. 3). Purified glycophorin A and the membrane protein components were stained with Coomassie blue (lanes 2 and 3, respectively). Glycophorin A was not susceptible to Coomassie blue staining as observed by others (Challou et al., 1994; Fairbanks et al., 1971; Furthmayr et al., 1975) and as seen by the absence of a band in lane 2. The glycoprotein bands appear in lanes 4 and 5, which are PAS-stained purified glycophorin A (lane 4) and membrane components (lane 5). Co-migration of glycophorin
inhibition of 8 units of HA antigen occurred with about 6 \( \mu \text{g} \) glycophorin A and inhibition totally disappeared at a glycophorin A concentration of about 0.4 \( \mu \text{g} \). These results suggest that the binding reaction between soluble glycophorin A and BPV masks the virus surface in such a way as to prevent virus attachment to its major erythrocyte receptor, the sialated glycoprotein glycophorin A.

**Discussion**

An important aspect of the biology of virus infection has to do with the molecular interactions of virus particles with the cell surface and the penetration of viruses into their host cells. Virus attachment to nucleated host cell receptors determines, in part, host range and tissue tropism. Although current evidence indicates that BPV replication does not occur in mature erythrocytes or erythrocyte progenitor cells, the attachment of virus to erythrocyte membrane glycoproteins is an important parameter of virus–cell interactions in the definition of overall BPV biology. Further, some parvovirus isolates replicate in erythrocyte progenitor cells, suggesting important interactions between viruses of this family and red blood cell membranes. The nature of the viral antireceptor, or the virus capsid, determines some extent the molecular mechanisms involved in virus attachment and penetration into the host cell. The results of the current study begin to define the interactions between BPV and membrane glycoproteins.

The 131 amino acid sequence of the glycophorin A monomer is arranged in such a way that the amino-terminal 61 amino acids compose the exoplasmic domain, amino acids 62 to 95 compose the hydrophilic transmembrane domain with the sequence from 73 to 95 forming an alpha helix, and amino acids 96 to 131 form the cytoplasmic domain. Both the exoplasmic domain and the cytoplasmic domain are rich in hydrophilic amino acids. The exoplasmic domain has 16 carbohydrate attachment sites; 15 carbohydrate chains are linked to serine or threonine residues and one longer one is linked to an asparagine residue. About sixty percent of the total molecular mass of glycophorin A is due to the carbohydrate side chains. In the erythrocyte membrane, glycophorin A is found as a dimer of two identical polypeptide monomers. These results suggest that the binding reaction between soluble glycophorin A and BPV masks the virus surface in such a way as to prevent virus attachment to its major erythrocyte receptor, the sialated glycoprotein glycophorin A.

![Graph showing inhibition of BPV-mediated HA with purified glycophorin A](image)

*Bovine parovirus erythrocyte receptors*

A is shown in these lanes. Using BPV as a virus probe on transblotted purified glycophorin A (lane 6) and membrane components (lane 7) and observing the virus-bound bands revealed by Western blotting, both co-migration of glycophorin A from membranes and purified material, and the ability of the virus to bind to glycophorin A from both sources were demonstrated.

Moreover, the purity of the glycophorin A preparation used throughout this study is confirmed by the absence of protein bands in lane 2, a single PAS-staining band in lane 4, and a single virus-binding band in lane 6.

Inhibition of HA with glycophorin A

Because the virus binds to glycophorin A, which is an integral part of the erythrocyte membrane, it can haemagglutinate erythrocytes by bridging the membranes of adjacent red blood cells, and ultimately form the red blood cell–virus lattice. However, it would be predicted that exposure of virus to soluble glycophorin A would bind to and mask the available attachment positions (antireceptors) on the virus structural proteins. Accordingly, experiments were performed in which various concentrations of virus (8, 4, 2 and 1 HA unit) were exposed to various concentrations of purified glycophorin A (twofold dilutions beginning with 12.5 \( \mu \text{g} \)) and the binding reaction was allowed to proceed. This was followed by the addition of human type O erythrocytes, incubation, and then wells were scored for HA. A series of HA controls without glycophorin A was included as well as glycophorin controls without virus. It was found that there was a concentration-dependent inhibition of HA by glycophorin A (Fig. 4). The results obtained in the experiment using 8 HA units are shown in Fig. 4, and as expected, glycophorin A similarly inhibited HA in the 4 unit, 2 unit and 1 unit experimental series. As the glycophorin A became less concentrated in a dilution series, less inhibition of virus-mediated HA occurred. Maximum
glycoproteins in the erythrocyte membrane to which BPV attaches in addition to glycophrin A. However, the glycophrin A complex from membranes was able to bind to the BPV particles. Purified glycophrin A in a dot blot assay was also active in binding BPV. Moreover, the SDS–polyacrylamide gel electrophoretic separation of membrane ghost components and purified glycophrin A, followed by Western blotting, showed that BPV would bind to both membrane and purified glycophrin A.

Some viruses, such as herpes simplex viruses, attach to heparan sulfate and, in fact, can attach to more than one molecular species of receptor molecule (for review see Norkin, 1995). Our competition study using purified glycophrin A to inhibit BPV-mediated HA provides insight into the possible ability of BPV to use more than one molecular species as receptor. It would be predicted that if BPV uses only sialic acid as a receptor, then purified glycophrin A would successfully compete with membrane receptors for BPV attachment. If BPV can bind to more than one type of receptor on the erythrocyte (other than molecules displaying sialic acid), purified glycophrin A should be unable to inhibit BPV-mediated HA completely. Since inhibition was complete and purified glycophrin A was able to compete with membrane receptors completely, it is unlikely that another different receptor type for BPV is available on the erythrocyte membrane.

Sialylglycoproteins associated with the membranes of nucleated cells may function as receptors for BPV initiating the virus replication cycle. Preliminary data indicate that exposure of virus to such glycoproteins inhibits BPV replication in buffalo lung cells.

The nature of the BPV antireceptor on the virus capsid, which interacts with the sialic acid receptor is unknown. However, some interesting information is known about the antireceptors of CPV and FPV, two closely related parvoviruses. The major structural protein of the capsid of CPV, VP2, configures in three monomers on the icosahedral triangular face. There is a depression or ‘dimple’ which spans the twofold axis of symmetry (the triangular edge). Both CPV and FPV haemagglutinate rhesus monkey and pig erythrocytes when they bind to sialic acid residues on these cells through a structure located in the dimple (Barbis et al., 1992). Although the BPV capsid contains three structural proteins and not two, as CPV and FPV, it would not be surprising to find a similar structural and functional role for the BPV major protein, VP3, but this has yet to be clarified.

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References


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