Mechanisms of bovine herpesvirus type 1 neutralization by monoclonal antibodies to glycoproteins gI, gIII and gIV

J. Dubuisson,† B. A. Israel and G. J. Letchworth III*

1Department of Virology-Immunology, Faculty of Veterinary Medicine, University of Liege, Institut de chimie (B6), 4000 Liege, Belgium and 2Department of Veterinary Science, University of Wisconsin-Madison, 1655 Linden Drive, Madison, Wisconsin 53706, U.S.A.

We examined a panel of monoclonal antibodies (MAbs) against bovine herpesvirus type 1 (BHV-1) glycoproteins gI, gIII and gIV for inhibition of virus attachment and interference with subsequent steps of infection. Attachment of radiolabelled virions was partially prevented by 600 to 700 μg/ml of IgM antibodies against gI and gIII and one IgG2A antibody against gIV, but not by the majority of MAbs against any of the three viral glycoproteins. Productive infection following attachment was prevented by lower concentrations of MAbs 5106 and 4807 against gI and by 0.7 to 5.5 μg/ml of all five MAbs against gIV. MAbs against gIV had almost the same activity whether added before or after BHV-1 was incubated with cells, suggesting that their principal activity is to prevent the penetration of virus through the cell membrane. The ability of polyethylene glycol to overcome neutralization by one anti-gIV MAb supported this concept, but an attempt to confirm this by direct electron microscopy failed. A bovine monospecific antiserum against gIV had approximately 10-fold more neutralizing activity against BHV-1 than did antisera against gI or gIII. Complement increased the activity of anti-gI and anti-gIII MAbs by 10- to 100-fold, but had little or no effect on neutralization by anti-gIV MAbs. Some antibodies against gI and gIV inhibited the enlargement of plaques in cell cultures. Taken together, these data suggest that MAbs against gIV are the principal agents of BHV-1 neutralization, and that these antibodies can be fully effective in areas such as the ocular and respiratory mucosae, from which complement is absent at the time of primary exposure to infection.

Introduction

The alphaherpesvirus bovine herpesvirus type 1 (BHV-1) is associated with a variety of clinical diseases including rhinotracheitis, conjunctivitis, reproductive tract lesions, encephalitis and generalized systemic infections (reviewed by Ludwig, 1982; Wyler et al., 1989), and as such is an economically significant pathogen of cattle (Gibbs & Rweyemamu, 1977; Yates, 1982). The BHV-1 envelope and the plasma membrane of infected cells contain a number of glycoproteins (Misra et al., 1981; van Drunen Littel-van den Hurk & Babiuk, 1986; Marshall et al., 1986), at least three of which, gI, gIII and gIV, appear to be involved in virus neutralization (van Drunen Littel-van den Hurk & Babiuk, 1986; Marshall et al., 1988). These three major envelope glycoproteins consist of three sets of coprecipitating polypeptides of apparent Mr, 130K/74K/55K (gI), 180K/97K (gIII) and 150K/77K (gIV) (van Drunen Littel-van den Hurk et al., 1984, 1985; van Drunen Littel-van den Hurk & Babiuk, 1985a, b, 1986; Chang et al., 1986; Collins et al., 1984; Okazaki et al., 1986; Marshall et al., 1986; reviewed by Wyler et al., 1989). Epitopes on each of these glycoproteins are targets for neutralizing monoclonal antibodies (MAbs) (Marshall et al., 1988). Competitive radioimmunoassays have mapped four interrelated epitopes on the 74K subunit of gI, three interrelated and two independent epitopes on gIV, and two interrelated and two independent epitopes on gIII. Each of these functional epitopes has been confirmed as a target for the bovine immune system during primary infection with BHV-1 (Marshall et al., 1988).

The neutralization of virus infectivity directly or indirectly by antibody has been proposed to be a major mechanism of immunological defence against virus invasion (Dimmock, 1984, 1987; McCullough, 1986), and several mechanisms, affecting early events of virus
infection, have been described (Dimmock, 1984, 1987; Iorio, 1988). Neutralizing antibodies can interfere with virus binding to specific cell surface receptors (Fuller & Spear, 1985; Ho et al., 1988; Weiss et al., 1988) by blocking the viral protein of attachment (Dimmock, 1987) or by aggregation of virions (Thomas et al., 1986). Antibodies can also neutralize enveloped viruses after attachment by inhibiting penetration. These antibodies inhibit fusion of the virion envelope with either the plasma membrane (Fuller & Spear, 1987) or a prelysosomal endosome after endocytosis of the virus (Gollins & Porterfield, 1986) depending on the mechanism of virus entry. Additionally, antibodies can neutralize virus subsequent to penetration by preventing uncoating (Dimmock, 1984), transcription of the genome (Possee et al., 1982; Kennedy-Stoskopf & Narayan, 1986) or virus-induced cell fusion (Noble et al., 1983; Minson et al., 1986).

The mechanisms of herpes simplex virus (HSV) neutralization have been extensively studied. Some MAbs to HSV gC and gD can block attachment of virus to cells (Fuller & Spear, 1985; Highlander et al., 1987). One neutralizing MAb to gD permits virus attachment, but prevents penetration of virus at the cell surface apparently by blocking fusion between the virion envelope and the plasma membrane (Fuller & Spear, 1987). Similar results have been obtained by using neutralizing MAbs to gB (Highlander et al., 1988) and to gH (Fuller et al., 1989). Antibodies against gB inhibit plaque development (Highlander et al., 1988). Other antibodies to HSV have also been shown to inhibit virus-induced cell fusion (Noble et al., 1983; Minson et al., 1986; Highlander et al., 1987).

BHV-1 and HSV belong to the same herpesvirus subfamily, and the BHV-1 gI, gII and gIV amino acid sequences are homologous to those of HSV gB, gC and gD respectively (Babiuk et al., 1988; Fitzpatrick et al., 1989; Misra et al., 1988; Tikoo et al., 1990; Whitbeck et al., 1988). BHV-1 gI can act as a fusion protein (Fitzpatrick et al., 1988), gIII appears to be the major attachment protein (Liang et al., 1991; Okazaki et al., 1991), and gIV is involved in virion penetration through the cell membrane (Chase et al., 1990; Tikoo et al., 1990) and cell fusion (Tikoo et al., 1990). It would be reasonable to suspect that each of these proteins is the target of neutralizing antibodies and that the mechanisms of BHV-1 neutralization closely parallel those of HSV. Indeed, some MAbs to gIII (gp87) block BHV-1 attachment, and some MAbs to gIV (gp71) interfere with the infection process subsequent to attachment (Okazaki et al., 1986; Hughes et al., 1988).

In this study MAbs to gI, gIII and gIV were used to define the mechanisms involved in the antibody-mediated neutralization of BHV-1. The glycoprotein-specific MAbs used in this study were directed against topographically different sites on gI, gIII and gIV (Marshall et al., 1988). By analyzing the specific mechanisms of neutralization for various antibodies we could show that virion attachment to cells is inhibited only at very high antibody concentrations, that productive infection following attachment is prevented by lower concentrations of antibodies against gI and gIV, that antibodies against gI and gIV inhibit the enlargement of plaques in cell cultures, and that complement increases the activity of anti-gI and anti-gIII MAbs by 10- to 100-fold, but has little or no effect on neutralization by anti-gIV antibodies. These data suggest that antibodies against gIV are the principal agents of BHV-1 neutralization and that these antibodies can be fully effective in areas such as the ocular and respiratory mucosa, from which complement is absent at the time of primary infection.

### Methods

**Cells and virus.** The Cooper-1 (Colorado-1) strain of BHV-1 was obtained from the ATCC (Rockville, Md., U.S.A.) and plaque-purified once. Virus was propagated on Madin-Darby bovine kidney (MDBK) cells (ATCC CCL22) grown in MEM (Gibco) supplemented with 5% foetal bovine serum (FBS) (Hyclone Laboratories). All the virus used in these experiments was at passage 14 to 16.

**MAbs.** The production of hybridomas, and the characterization of the MAbs used in this work and the topology of their corresponding epitopes have been described elsewhere (Marshall et al., 1986, 1988). MAbs were purified by ammonium sulphate precipitation from ascites fluids obtained from BALB/c mice bearing intraperitoneal hybridomas. Precipitated antibodies were analysed by SDS-PAGE and Coomassie blue staining to show that the majority of protein was indeed MAb.

**Antiserum and complement.** Monospecific antiserum to individual BHV-1 envelope proteins were prepared by immunizing BHV-1-seronegative calves intradermally with glycoproteins purified on MAb affinity columns. The presence of antibodies to epitopes known to be targets for neutralization was determined by competitive radioimmunoassay and is described elsewhere (Israel et al., 1988). Convalescent serum collected from a calf 17 days after BHV-1 infection was used as polyvalent antiserum. Neutralizing antigenic areas recognized by the serum have been determined previously (Marshall et al., 1988). Guinea-pig serum (as a complement source) was collected fresh in-house and was flash-frozen in liquid nitrogen. It was used at the dilution giving maximum enhancement of neutralization without cytotoxicity.

**Preparation of purified labelled virus.** MDBK cells grown to confluence were infected with BHV-1 at a multiplicity of 3 p.f.u. per cell and incubated for 6 h at 37 °C in MEM with 5% FBS. Infected cells were washed twice with methionine-free MEM, and methionine-free medium containing 25 μCi of [35S]methionine (New England Nuclear)/ml was added. After 40 h of incubation, virus was purified from the supernatant by centrifugation through a cushion of 30% sucrose dissolved in PBS (0.15 M-NaCl, 2.9 mM-KCl, 89 mM-Na2HPO4, 1.6 mM-KH2PO4, pH 7.2) and two successive sediments through a potassium tartrate gradient (20 to 50% potassium tartrate in PBS pH 7.2) as described by Misra et al. (1981).
Inhibition of attachment assay. Virus attachment was measured by using radiolabelled virus following the procedure of Fuller & Spear (1985). Briefly, MDBK cells growing as confluent monolayers in 96-well plates were incubated with PBS containing 1% BSA solution, 1% inactivated calf serum and 0-1% glucose at 4 °C. Purified radiolabelled virus was incubated for 1 h at 37 °C with various concentrations (8, 40, 200 and 1000 μg/ml) of different MAbs to BHV-1 or an irrelevant MAb. The 96-well plates were incubated with the antibody-virus mixture (1 × 10^6 p.f.u./well, approximately 3000 virus particles/cell) at 4 °C for 2 h to allow attachment of virus to cells. The supernatant was removed and the wells were washed at least three times. Cells and bound virus were removed from the wells by solubilization in PBS containing 1% Triton X-100 and 1% SDS. The c.p.m. bound to cells was calculated from the average of triplicate samples. The percentage c.p.m. bound to cells was calculated as the ratio of c.p.m. bound in the presence of MAb to BHV-1 to c.p.m. bound in the presence of an irrelevant MAb. These values were plotted and the concentration of antibody reducing virion attachment by 50% was calculated by interpolating from the graph. These measurements were done both in the presence and absence of active guinea-pig complement.

Post-adsorption and pre-adsorption neutralization. For the post-adsorption neutralization, MDBK cells growing as confluent monolayers in 96-well plates were infected with 100 TCID<sub>50</sub> BHV-1/well and incubated for 2 h at 4 °C to allow virus to attach to the cells. Infected cells were washed once with PBS, and 100 μl of twofold dilutions of individual MAbs beginning at 1000 μg/ml was added for an additional 2 h at 4 °C. At the same time a pre-adsorption neutralization test (classical neutralization test) was performed for comparison. BHV-1 (200 TCID<sub>50</sub>) from the same stock solution was mixed with an equal volume of twofold dilutions of individual MAbs beginning at 2000 μg/ml and incubated for 2 h at 4 °C. Then antibody-virus mixtures (100 μl/well) were added to duplicate MDBK cell monolayers in 96-well plates and incubated at 4 °C for 2 h. The monolayers were fixed and stained with crystal violet after 6 days of incubation at 37 °C. Fifty percent endpoint titres were calculated by the method of Reed & Muench (1938). These experiments were performed both with and without active guinea-pig complement. An irrelevant MAb was used as a control. Pre-adsorption and post-adsorption virus neutralization were tested with antisera to BHV-1 and monospecific antisera to BHV-1 glycoproteins gI, gIII and gIV following the same procedure. Differences between post-adsorption and pre-adsorption neutralization titres were considered significant if P < 0.05 by Student's t-test.

Antibody-mediated alteration of plaque development. Confluent MDBK cells grown in six-well plates were infected with BHV-1 at 250 to 330 p.f.u./well and incubated for 2 h at 37 °C in MEM containing 5% FBS. Cells were washed with PBS and overlaid with MEM containing 5% FBS, diluted MAb (1, 10, 50 and 100 μg/ml) and 0-5% methylcellulose. An irrelevant MAb was used as a control in this experiment and all the MAbs were tested at the same time. Antisera to BHV-1 and BHV-1 glycoproteins gI, gIII and gIV were tested at 1:10, 1:100 and 1:1000 dilutions following the same procedure. The monolayers were fixed and stained after 5 days. Plates were scored with a grid and the diameter of the first 25 isolated single plaques in one grid space was measured with an ocular micrometer. Mean plaque diameters were considered significantly different if P < 0.05 by Student's t-test.

Restoration of infectivity with polyethylene glycol (PEG). Concentrated BHV-1 was incubated for 1 h at 37 °C with 1000 μg/ml of MAb or with the same volume of MEM with or without complement, and 10-fold dilutions of the mixture were plated in duplicate on confluent MDBK cells grown in 24-well plates. After 1 h attachment at 37 °C, one of the duplicates was exposed to PEG as described by Sarmiento et al. (1979) and Little et al. (1981). After treatment with PEG, the cultures were overlaid with MEM containing 5% FBS and 0-75% methylcellulose, and incubated at 37 °C for 4 days. Monolayers were fixed and stained, and plaques were counted.

Electron microscopy. To visualize the effect of antibodies on virus attachment to cell membranes, BHV-1 at 2 × 10<sup>8</sup> p.f.u./ml was incubated with ammonium sulphate-precipitated MAbs (1000 μg/ml), or with undiluted bovine antisera for 1 h at 37 °C. MDBK cells grown on glass coverslips were pretreated with MEM containing 1 mm-HEPES and 1% heat-inactivated foetal calf serum for 10 min at 4 °C. The cells were incubated with the antibody-virus mixture at 4 °C for 2 h. The mixture was removed and the monolayers were washed three times with PBS before fixation for 15 min in Karnovsky's fixative (1:25% glutaraldehyde and 2% paraformaldehyde in 0-1 m-phosphate buffer). The cells were rinsed once and stored in 0-1 m-phosphate buffer at 4 °C until processing for electron microscopy. Cells were embedded in situ and sections were cut from the apical surface. Sections were stained with uranyl acetate and lead citrate, and examined with a Phillips 410 electron microscope. Virions adherent to the cell membrane of 15 to 20 well-isolated cells were counted. Mean virion counts were considered significantly different if P < 0.05 by Student's t-test.

The ability of MAbs to interfere with virus penetration was examined by treating virions both before and after attachment to the cell membrane. Virus and antibody were incubated for 1 h at 37 °C before inoculation onto pretreated monolayers of MDBK cells. Infection was allowed to proceed for 30 min at 37 °C, after which the cells were washed and fixed as described above. Alternatively, virus was inoculated onto pretreated monolayers of MDBK cells at 4 °C for 2 h to allow attachment to cell membranes. MAbs or polyvalent bovine antisera were added to a final concentration of 1000 μg/ml and the cells were incubated for an additional 2 h at 4 °C. The cells were then incubated at 37 °C for 30 min to allow penetration to proceed. Cells were fixed, embedded, sectioned and examined as described previously. Virions adherent to the cell membrane of 15 to 20 isolated cells were counted.

Results

Inhibition of attachment

The binding of purified radiolabelled virus to MDBK cells was determined at 4 °C to measure virus attachment under conditions that prevented virus penetration. Non-specific interactions of virus with the surface of the microtitre plate and with the cells were minimized by pretreatment of monolayers with BSA. Table 1 shows the concentration of MAbs required to reduce the binding of radiolabelled BHV-1 by 50% (1-5 × 10<sup>6</sup> p.f.u./ml) in the presence or absence of complement. In all these experiments, the deviation of each value from the mean was no more than 15%. MAbs 5606 (IgM, anti-gI), 0922 (IgM, anti-gIII) and 3402 (IgG2A, anti-gIV) inhibited BHV-1 attachment at high concentrations (708 μg/ml, 691 μg/ml and 620 μg/ml, respectively) in the absence of complement. In the presence of complement, these MAbs also inhibited BHV-1 attachment but at a lower concentration and two additional MAbs, 1507 (IgG2A, anti-gIII) and 0722 (IgG2A, anti-gIV), also inhibited BHV-1 attachment. Some MAbs reduced binding of
radiolabelled purified BHV-1 by less than 50% (between 24% and 46%) at a MAb concentration of 1000 μg/ml (MAbs 4807 and 4203 with or without complement, and MAbs 1106, 4906 and 1102 only in the presence of complement). Other MAbs increased virus binding to cells two- to fourfold in the presence (MAbs 5106 and 3002) or the absence (MAbs 2905, 1802, 0722, 1106, 4906 and 1102) of complement (data not shown). Electron microscopy confirmed that virions treated with MAb 3402 (directed against gI/IV, antigenic area V) before inoculation onto MDBK monolayers were prevented from adsorbing to the cell membrane. A mean of 14.3 virions was counted on the cell membrane of control cells. None were seen on cells treated with BHV-1 and MAb 3402. The absence of truly random samples in this experiment precluded statistical evaluation.

Post-adsorption and pre-adsorption neutralization

Some neutralizing MAbs to BHV-1 did not inhibit virus attachment to cells. It is possible that such antibodies block a step in infectivity subsequent to virus attachment, such as the entry of virions into the cell cytoplasm. Antibodies that function at a post-attachment step can be identified by their ability to neutralize with similar efficiencies both free virus and virus already bound to cell membranes. Therefore, to explore this mechanism of neutralization, MAbs to gI, gII and gIV and antisera to individual glycoproteins were compared for their ability to neutralize virus before and after attachment to the host cell.

Some anti-gI and anti-gIV MAbs neutralized BHV-1 at similar concentrations both before and after attachment, and the effectiveness of the others was four- to eightfold greater before attachment (Table 1). Anti-gII MAbs neutralized BHV-1 only before attachment, only in the presence of complement, and only at relatively high antibody concentrations (Table 1). For some MAbs (gI antigenic site IV and gII antigenic sites), the 50% neutralization concentration could not be measured accurately because a linear relationship did not exist between the concentration of antibody used and the appearance of c.p.e. This problem has been noted previously (Marshall et al., 1988).

We used PEG treatment of neutralized virus to show that neutralized virions were simply precluded from penetration into cells. Virions were neutralized and incubated on MDBK cells, and the cells were then treated with PEG. As shown in Fig. 1, PEG treatment of MDBK cells infected with 10-fold dilutions of virus neutralized with MAb 1106 (directed against gIV) increased the infectious titre 100-fold. It is probable that the recovery of only about 0.1% of the virus is caused by endocytosis and degradation of the majority of the antibody-coated particles. Virus was not recovered if it had been treated with MAb 1106 in the presence of complement. We attempted to confirm by electron microscopy that virions treated with MAb 1106 could

<table>
<thead>
<tr>
<th>BHV-1 gp</th>
<th>Antigenic area*</th>
<th>MAb*</th>
<th>Radiolabelled BHV-1</th>
<th>50% Inhibition of attachment</th>
<th>50% Neutralization</th>
<th>50% Inhibition of penetration by electron microscopy</th>
<th>Statistically significant inhibition of plaque growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>gI</td>
<td>I 5106 IgG2A</td>
<td>&lt;1000</td>
<td>1000*</td>
<td>45</td>
<td>35</td>
<td>5.5</td>
<td>ND§</td>
</tr>
<tr>
<td></td>
<td>II 4807 IgG2B</td>
<td>&gt;1000</td>
<td>&gt;1000*</td>
<td>91</td>
<td>91</td>
<td>91</td>
<td>ND§</td>
</tr>
<tr>
<td></td>
<td>III 5606 IgM</td>
<td>&gt;1000</td>
<td>&gt;1000*</td>
<td>&gt;452</td>
<td>5.5</td>
<td>5.5</td>
<td>ND§</td>
</tr>
<tr>
<td></td>
<td>IV 4203 IgG2A</td>
<td>&gt;1000</td>
<td>&gt;1000*</td>
<td>&gt;1000*</td>
<td>45</td>
<td>45</td>
<td>ND§</td>
</tr>
<tr>
<td>gII</td>
<td>I 1507 IgG2A</td>
<td>&gt;1000</td>
<td>40</td>
<td>&gt;91*</td>
<td>45</td>
<td>91</td>
<td>ND§</td>
</tr>
<tr>
<td></td>
<td>II 0922 IgM</td>
<td>691</td>
<td>140</td>
<td>1000</td>
<td>91</td>
<td>&gt;1000*</td>
<td>ND§</td>
</tr>
<tr>
<td></td>
<td>III 2905 IgG2A</td>
<td>&gt;1000</td>
<td>&gt;1000*</td>
<td>&gt;1000*</td>
<td>11.5</td>
<td>&gt;1000*</td>
<td>ND§</td>
</tr>
<tr>
<td></td>
<td>IV 3902 IgG1</td>
<td>&gt;1000</td>
<td>&gt;1000*</td>
<td>&gt;1000*</td>
<td>91</td>
<td>&gt;1000*</td>
<td>ND§</td>
</tr>
<tr>
<td></td>
<td>V 1808 IgG2A</td>
<td>&gt;1000</td>
<td>&gt;1000*</td>
<td>&gt;1000*</td>
<td>11.5</td>
<td>&gt;1000*</td>
<td>ND§</td>
</tr>
<tr>
<td>gIV</td>
<td>I 0722 IgG2A</td>
<td>&gt;1000</td>
<td>862</td>
<td>0-7</td>
<td>0-7</td>
<td>2.8</td>
<td>1-4</td>
</tr>
<tr>
<td></td>
<td>II 1106 IgG2A</td>
<td>&gt;1000</td>
<td>&gt;1000*</td>
<td>0-7</td>
<td>0-7</td>
<td>&gt;1000*</td>
<td>ND§</td>
</tr>
<tr>
<td></td>
<td>III 4906 IgG2A</td>
<td>&gt;1000</td>
<td>&gt;1000*</td>
<td>5-5</td>
<td>2-8</td>
<td>2-8</td>
<td>ND§</td>
</tr>
<tr>
<td></td>
<td>IV 1102 IgG2A</td>
<td>&gt;1000</td>
<td>&gt;1000*</td>
<td>ND§</td>
<td>ND§</td>
<td>11-5</td>
<td>ND§</td>
</tr>
<tr>
<td></td>
<td>V 3402 IgG2A</td>
<td>&gt;1000</td>
<td>&gt;1000*</td>
<td>2-8</td>
<td>2-8</td>
<td>2-8</td>
<td>ND§</td>
</tr>
</tbody>
</table>

* Data from Marshall et al. (1988).
† Cplt, Complement.
§ ND, Not determined.
| Confirmed by electron microscopy with MAb 3402 at 1 mg/ml.
Table 2. Biological activities of bovine antisera to BHV-1 and to isolated BHV-1 glycoproteins gI, gIII and gIV

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Antigenic area*</th>
<th>Neutralization titres</th>
<th>Inhibition of plaque enlargement†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before attachment‡</td>
<td>After attachment‡</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cplt − §</td>
<td>Cplt +</td>
<td>Cplt −</td>
</tr>
<tr>
<td>gI</td>
<td>I, II, III, IV</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>gII</td>
<td>I, IV, gI (II)</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>gIV</td>
<td>I, II, III, IV, V</td>
<td>64</td>
<td>128</td>
</tr>
<tr>
<td>BHV-1</td>
<td>gI (I, II, III, IV)</td>
<td>64</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td>gIII (I, V)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>gIV (I, II, IV)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Data from Israel et al. (1988) and Marshall et al. (1988).
† Infected cells were incubated with 1/10, 1/100 and 1/1000 dilutions of antisera.
‡ Titres are recorded as the reciprocal of the highest MAb dilution yielding more than 25% c.p.e.
§ Cplt, Complement.

Antibody alteration of plaque development

To investigate whether neutralizing MAbs affected the ability of gI, gIII or gIV to mediate the membrane-related events required for the spread of virus from infected to uninfected cells, monolayers of MDBK cells were infected with BHV-1 and incubated under methylcellulose containing various concentrations of MAbs and monospecific antibodies to gI, gIII and gIV, or an antiserum to BHV-1. Two MAbs to gI (5106 and 4807) and two MAbs to gIV (1102 and 3402) (Table 1) significantly reduced plaque size to 61, 77, 53 and 64% of control diameters respectively. MAb 5106 also reduced the total number of plaques to 89% of the control value (253 against 283 plaques/well), suggesting that some plaques might have been reduced below microscopically observable size. Bovine monospecific antiserum to gIV significantly reduced plaque size to 83% of that of the negative control. Other bovine antisera had no effect.

Discussion

The exact molecular mechanism of alphaherpesvirus infection and the means by which antibodies interfere with this process are not completely clear. The purpose of this study was to determine the mechanisms by which gI-, gII- and gIV-specific antibodies neutralize BHV-1. Previous studies of BHV-1 and its human homologue HSV show that gIII is the main attachment protein whereas gI and gIV play minor roles in attachment but are critical for penetration through the cell membrane. Therefore, antibodies against gIII might be expected to interfere with attachment, and antibodies against gI and
gIV should preferentially inhibit post-attachment events. Complement should be able to interact with most antibodies to envelop the virion in a thick blanket of protein that prevents interaction of viral with cellular molecules. Our data fit this picture relatively well. These investigations provide information about the molecular targets and mechanism of action of neutralizing antibodies, and clarify the role of complement in BHV-1 neutralization. Additionally, these studies have probed the molecular interactions leading to virus entry into cells and spread between cells. The information obtained should help direct the development of more effective BHV-1 vaccines.

HSV gC appears to be involved in virion attachment to cells because some MAbs to HSV gC neutralize the virus (Fuller & Spear, 1985), and some both neutralize and block attachment of virus to cells (Langeland et al., 1990). Based on the result that isolated gIII (gp87) selectively attaches to susceptible cells and that one group of MAbs to gIII inhibits BHV-1 attachment to cells, Okazaki et al. (1987) postulated that gIII is the principal agent of virus attachment. This has twice been confirmed (Liang et al., 1991; Okazaki et al., 1991) and is consistent with similar findings in HSV (Herold et al., 1991). However, gIII is not required for virus infection (Liang et al., 1991), presumably because other viral glycoproteins can substitute for gIII in virion attachment. Thus it is not surprising that only one of the five MAbs to gIII (0922) inhibits attachment of radiolabelled virus in the absence of complement and only at a very high antibody concentration (Table 1). This is an IgM MAb and probably inhibits attachment of the virus to cells by steric hindrance. The addition of complement probably increases this hindrance. This is consistent with the 10- to 100-fold reduction in virus titres when complement was added to the mixture of BHV-1 and any of the five MAbs against gIII, and the observation that anti-gIII antibodies with or without complement have no effect on virus that has already attached to the cell membrane. Although less likely, this also could be explained by an allosteric modification of gIII after virus attachment preventing recognition by these MAbs after BHV-1 attachment. A bovine monospecific antiserum containing antibodies to two gIII epitopes and one gI epitope was able to neutralize BHV-1 after attachment and to neutralize in the absence of complement (Table 2). Several explanations are possible. This activity may have been due to the anti-gI activity in this antiserum, or the BHV-1 neutralization mechanisms acting against gIII in the natural host differ from those observed with individual MAbs to gIII. Also, there could be synergistic action between different antibodies or antibody isotypes that neutralize by different mechanisms (Dimmock, 1984, 1987). Alternatively, the bovine serum may contain antibodies to functional epitopes on the gIII molecule that are not recognized by our collection of MAbs. Since none of these alternative explanations was explored, firm conclusions are impossible. Nevertheless, the titre of anti-gIII neutralizing antibody was very low. Taken together, these results suggest that gIII functions only early in the infection process, is not critical to that process, and is not a useful target for neutralizing antibodies.

Complement increases the ability of two of five anti-gIII MAbs to inhibit virus attachment and dramatically increases the ability of all five to inhibit infection. Complement can enhance virus neutralization by envelopment of the virus or aggregation of virus–antibody complexes, and probably can inhibit virus attachment or penetration by steric interference (Wallis & Melnick, 1971). Conversely, complement can solubilize virus–antibody complexes (Miller & Nussenzweig, 1975). Although it is possible that prevention of attachment by MAbs and complement is an illusion created by virolysis and release of radiolabelled proteins, this is unlikely because herpesvirus neutralization occurs mainly by deposition of a thick layer of antibody and complement on the virion, presumably burying the viral attachment proteins (Cooper & Nemerow, 1984).

HSV gB interacts with cell membranes (Kühn et al., 1990) where it functions in virion penetration (Sarmiento et al., 1979; Little et al., 1981; Cai et al., 1988) and participates in membrane fusion (Ali et al., 1987; Cai et al., 1988). HSV gB is not essential for virion attachment (Cai et al., 1988) and one MAb to gB neutralizes HSV after virion attachment in the absence of complement (Highlander et al., 1988). The closely homologous BHV-1 gI also acts as a fusion protein in murine (Fitzpatrick et al., 1990) and bovine (Tikoo et al., 1990) cells. Thus it is not surprising that only one of our four anti-gI MAbs partially inhibits attachment of BHV-1 to cells and only at a high concentration (Table 1). Complement increases this activity 7-3-fold, probably by steric hindrance. Two anti-gI MAbs neutralize BHV-1 both before and after attachment to cell membranes, suggesting that these antibodies interfere with a post-attachment process. Similar results were obtained with bovine antisera to gI. Complement increases the activity of anti-gI MAbs in three of four cases, and more than 100-fold in one case (MAb 5106). This is difficult to interpret because these three antibodies have strong complement lysis activity (Marshall et al., 1988) and thus may have been acting either on the virions or on the infected cells, or both. Anti-gI MAb 5106 also inhibits plaque enlargement. These data suggest that the mechanism of BHV-1 neutralization acting on gI mainly involves inhibition of virion penetration and cell-to-cell fusion, and that gI could play a role in a BHV-1 vaccine. These data also
suggest that BHV-1 gI, like its homologue HSV gB, may normally function in virion penetration.

The mechanisms of neutralization of MAbs to gIV are very similar to those observed for the homologous HSV gD. HSV gD interacts with cell membranes (Kühn et al., 1990; Johnson et al., 1990) where it functions in virion penetration (Ligas & Johnson, 1988). Mechanisms of HSV neutralization by anti-gD MAbs have been studied extensively (Para et al., 1985). Antibodies directed against HSV gD are more efficient in neutralization than antibodies directed against other viral glycoproteins. Some neutralizing anti-gD MAbs inhibit virus attachment (Fuller & Spear, 1985), some inhibit cell fusion (Noble et al., 1983), and one MAb to gD that permits virus attachment has been shown to prevent penetration of virus at the cell surface by blocking fusion between the virion envelope and the plasma membrane of cells (Fuller & Spear, 1987). The same mechanism has been suggested for other MAbs to gD (Highlander et al., 1987).

One of the five MAbs to gIV (3402) inhibits BHV-1 attachment to MDBK cells in the absence of complement. This was shown by interference with the binding of radiolabelled virus and supported by direct observation in electron microscopy. This supports the role of gIV in BHV-1 attachment to the cell membrane.

More importantly, very low concentrations of all anti-gIV antibodies either in the presence or absence of complement neutralize BHV-1 both before and after virion attachment to cells. This argues strongly against neutralization by aggregation and suggests that anti-gIV antibodies act mainly by blocking a step subsequent to attachment. Virus neutralized by MAb 1106 after attachment to cells could be restored to infectivity by PEG treatment. This suggests that neutralized virus stays on the cell surface and that PEG treatment forces virion penetration by inducing fusion of the virion envelope with the plasma membrane, as shown for HSV (Fuller & Spear, 1987; Fuller et al., 1989). We attempted to confirm this by electron microscopy. Virions treated with MAb 1106 could attach to the cell membrane and remained there despite incubation at 37 °C, but virus treated with negative control antibody also remained on the cell surface, preventing meaningful comparison. PEG treatment had no effect on virus neutralized by MAb 1106 in the presence of complement. This may be explained by the physical envelopment of the virion in complement (Cooper & Nemerrow, 1984). Okazaki et al. (1986) and Hughes et al. (1988) have previously described MAbs to gI (gp71) that neutralize infectivity after virus attachment, but the low antibody concentrations required, the diversity of epitopes involved, and the probable mechanism of action have not been determined.

The epitope recognized by MAb 3402 may be situated in a region of the gIV glycoprotein that is critical to its functional interaction with membranes and membrane-bound molecules. This antibody inhibited virus attachment, neutralized virus before and after virion attachment to cells, and decreased the enlargement of plaques. PEG treatment did not restore the infectivity of virus neutralized by MAb 3402, suggesting that this MAb can irreversibly neutralize BHV-1 in a step before penetration. Competitive radioimmunoassays with a panel of MAbs directed against gIV suggest the antigenic area recognized by MAb 3402 is independent of the other four antigenic areas (Marshall et al., 1988). The amino acid sequence or relative position of these antigenic areas on gIV is not known.

A bovine monospecific antiserum against gI and a bovine polyspecific antiserum against BHV-1 virions, both containing antibodies to all five epitopes on gIV, were also able to neutralize BHV-1 after attachment at titres similar to those obtained when the neutralization test was performed before attachment. Together these data show that anti-gIV antibodies neutralize BHV-1 primarily by interfering with a process that follows virion attachment.

The inhibition of plaque enlargement by anti-gI MAbs 5106 and 4807 and anti-gIV MAbs 1102 and 3402 can be explained by interference with the hypothetical formation of intercellular bridges that allow BHV-1 to pass from infected cells to uninfected cells without release into the medium. Such a process is proposed for some neutralizing antibodies against HSV gB and gD (Minson et al., 1986). Alternatively, anti-BHV-1 antibodies might prevent virus release from infected cells. Örvell & Kristensson (1985) have shown that antibodies to Sendai virus haemagglutinin-neuraminidase inhibit virion release from infected cells. These antibodies cause aggregation of virions in contact with the plasma membrane, as shown by electron microscopy, but the antibodies do not prevent cell-to-cell spread of infection. We have no evidence for such a mechanism in BHV-1 neutralization. Whatever the mechanism, inhibition of plaque enlargement may not be important for control of the virus in the natural host. Indeed, antibodies in bovine sera to gI, gIV and whole virus recognize epitopes involved in plaque size reduction, but only the antisera against gIV significantly inhibit plaque development.

Protection against BHV-1 involves more than neutralizing antibodies circulating in the plasma. Marshall & Letchworth (1988) tested calves for passive protection engendered by each of the MAbs used in the present work. Despite attaining high circulating levels, none of the MAbs affected subsequent BHV-1 infection or disease. This suggests that protection requires neutralizing antibodies at the site of infection, i.e. mucosal surfaces, rather than just circulating in the plasma. Since
complement is not available on mucosal surfaces, the antibodies most likely to provide protection are those which can neutralize virus in the absence of complement. Only antibodies against gI and gIV appear to be 10- to 100-fold more effective than those against gII. Nevertheless, Israel et al. (1992) have shown that even a gI intranasal vaccine can induce protective antibodies in the nasal secretions of cattle.

J. Dubuisson was research assistant of the ‘Fonds National Belge de la Recherche Scientifique’. Thanks are due to Dr E. Thiry for helpful advice, and to K. Carter-Allen, R. Herber and R. Bromberg for excellent technical assistance. This work was supported by USDA grant no. 86-CSRS-2-2902 and a Shaw Scholarship to G.J.L. from Milwaukee Foundation.

References


(Received 4 December 1991; Accepted 24 April 1992)