Functional and topographical analyses of epitopes on bovine herpesvirus type 1 glycoprotein IV

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Summary. Bovine herpesvirus type 1 (BHV-1) glycoprotein gIV was purified by affinity chromatography. Purified preparations showed two distinct components of 71 K and 140 K following electrophoresis in sodium dodecyl sulphate poly-acrylamide gels. The polypeptides were separated, excised from the gel and used to immunize rabbits; the resulting antisera showed a high degree of cross reactivity indicating that these polypeptides represent monomeric and dimeric forms of the same glycoprotein. Purified gIV was also used to develop a gIV-specific panel of monoclonal antibodies. Neutralizing monoclonal antibodies directed against gIV were conjugated to horseradish peroxidase and subjected to competition binding assays by ELISA. Three distinct neutralizing antigenic domains on gIV were identified. Domain 1 comprised two overlapping epitopes, whereas domain 2 was represented by a single monoclonal antibody. The third antigenic domain was made up of a complex of four identical or overlapping epitopes designated 3 a, b, c, and d. Evidence is presented suggesting that domain 1 of gIV may be involved in penetration of the virus into the cell.

Introduction

Bovine herpesvirus type 1 (BHV-1), a member of the alphaherpesvirinae, has been shown to express three major glycoproteins on the surface of infected cells and the viral envelope [19]. These glycoproteins, designated gI (130 K, cleaved to give 74 K and 55 K), gIII (91 K with a 180 K dimer), and gIV (71 K) are thought to be of major importance in host response to infection and in virus attachment and entry into the cell. Earlier studies utilizing affinity-purified glycoproteins as subunit vaccines have shown that all three glycoproteins can induce protection from a lethal challenge with BHV-1 followed by *Pasteurella haemolytica*, with gIV appearing to be of major importance in this regard [1].

The range of glycoproteins identified in herpes simplex virus (HSV) is much wider numbering seven to date [13]. The three BHV-1 glycoproteins (gI, gIII,

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and gIV) have been shown to be analogous to glycoproteins in HSV: gI to gB of HSV, gIII to HSV gC and gIV to HSV gD [Zamb, manuscript in preparation]. HSV-1 gD induces high titres of virus neutralizing antibodies and is able to protect animals from experimental challenge [6, 11, 12]. In HSV, gD is thought to be involved in virus penetration [3, 8].

Thus far, we obtained one gIV-specific monoclonal antibody following immunization with purified BHV-1 [16]. In order to study the role of gIV in BHV-1 infection more fully, a panel of monoclonal antibodies was generated against affinity-purified gIV. The resulting neutralizing monoclonal antibodies were used to identify discrete epitopes of gIV on the surface of virions. Similar studies involving other glycoproteins of BHV-1 have revealed one neutralizing antigenic domain on gI and two on gIII using one panel of monoclonal antibodies [5]. Six neutralizing epitopes and one non-neutralizing epitope on gI as well as one neutralizing epitope on gIII were identified [18] with a different panel of monoclonal antibodies. Three neutralizing antigenic domains on gIII and one on gIV with a panel of monoclonal antibodies have been identified independently [15].

In this study the neutralizing monoclonal antibodies obtained were used to try to elucidate the function of gIV of BHV-1. To date no specific function has been assigned to this glycoprotein in BHV-1 though recent studies with HSV [3, 8] suggest that the equivalent glycoprotein (gD) is involved in virus penetration of the host cell.

Materials and methods

Viruses and cells

The P8-2 strain of BHV-1 was propagated in Georgia Bovine Kidney cells (GBK cells) grown as monolayers in Eagle's Minimal Essential Medium (MEM) (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Grand Island Biological Co.).

Virus purification

BHV-1 was purified from the culture medium of rapidly dividing GBK cells infected at 24 h post inoculation with a multiplicity of infection (moi) of 1. Following low speed and high speed centrifugation (5 minutes at 5 g and 90 minutes at 83,000 g, respectively) the virus pellets were centrifuged through potassium tartrate gradients as described previously [17].

Monoclonal antibodies

Glycoprotein gIV was purified on an immunoadsorbent column consisting of monoclonal antibody 3D9S [1, 16], linked to Affigel-10. BALB/c mice (Jackson Laboratories, Bar Harbour, ME) were injected intraperitoneally with 0.1 ml (50 μg) of purified gIV mixed by sonication with an equal volume of Freund's complete adjuvant. One week later the mice were inoculated intraperitoneally with 50 μg of gIV in Freund's incomplete adjuvant. This was repeated after two weeks and three weeks. The final immunization on day 35 consisted of 50 μg of gIV in phosphate buffered saline inoculated into the tail vein. Fusion was

performed three days later by a modification of the method of Kennett et al. [9]. The supernatants of growing hybridoma cell lines were screened for gIV-specificity using an ELISA with purified BHV-1 virions as the antigen. Clones showing the required specificity were subcloned in microtitre plates using the limiting dilution method.

Ascites fluids were obtained by intraperitoneal inoculation of 10^6 to 10^7 specific anti-body-producing hybridoma cells into BALB/c mice that had been primed 2–3 weeks previously with pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemicals, Montreal, Quebec).

Preparation of monospecific rabbit antisera

Glycoprotein gIV was purified by immunoaffinity chromatography, followed by preparative sodium dodecyl sulphate polyacrylamide gel electrophoresis. The 71 K and 140 K glyco-protein bands were excised from the gel, emulsified in PBS and an equal volume of Freund's complete adjuvant. New Zealand white rabbits were given one initial immunization of each of the glycoproteins in Freund's complete adjuvant, followed by two subsequent immu-nizations with Freund's incomplete adjuvant at 4-week intervals. The rabbits were bled approximately 3 weeks after each immunization.

Virus neutralization assays

The ability of the hybridoma culture supernatants to mediate virus neutralization was tested as described previously [17] using 2-fold dilutions in the absence and presence of guinea pig serum (at 1/40 dilution) to determine complement-enhanced neutralization. Ascites fluids were tested for their neutralization titres which were judged to be the highest dilution resulting in a 50% reduction of plaques relative to a virus control.

Post-adsorption virus neutralization assays

The monoclonal antibodies were tested for their ability to neutralize virus after viral attachment by a modification of the standard neutralization assay. Virus was adsorbed to cell monolayers in 96-well plates for 2 h at 4 °C and incubated with dilutions of the mono-clonal antibodies for a further 2 h at 4 °C. Finally the monoclonal antibody was removed and replaced with MEM containing 2% FBS and incubated at 37 °C for approximately 36 h. The monolayers were then stained and the levels of neutralization determined as a 50% reduction in plaques relative to a virus control.

Antibody and complement-mediated cytolysis (AbC)

Complement-dependent cell lysis was determined as described previously [2].

Preparation of horseradish peroxidase (HRPO)-conjugated monoclonal antibodies

The HRPO conjugated monoclonal antibodies were prepared as previously described [18]. Briefly, immunoglobulin was precipitated from ascites fluid by 50% $(NH_4)_2SO_4$ overnight, and the pellets were collected and desalted on PD 10 columns (Pharmacia, Montreal, Quebec) in 0.01 M $NaHCO_3/Na_2CO_3$ pH 9.6. The IgG was then incubated for 3 h at 22 °C with activated HRPO at a ratio of 30 mg IgG to 10 mg HRPO. The reaction was halted by the addition of 10 mg $NaBH_4$ and the conjugates were dialyzed overnight at 0 °C in phosphate buffered saline (PBS: 0.01 M NaH_2PO_4/Na_2HPO_4, 0.15 M NaCl pH 7.4).

Enzyme-linked immunosorbent assay (ELISA)

Microtitre plates (Immulon 2, Dynatech Laboratories Inc., Alexandria, VA) were coated with 200 µl/well of BHV-1 at 10 µg/ml in carbonate/bicarbonate buffer (0.05 M $NaHCO_3/Na_2CO_3$ pH 9.6) at 37 °C for 2 h or 4 °C overnight. Plates were washed six times in PBS with

0.05% TWEEN 20 (PBST). Monoclonal antibodies were screened at a 1/10 dilution in PBST (200 µl/well), and ascites fluids were tested to their dilution end points. After incubation for 2 h at 37 °C, the plates were again washed six times in PBST, they were then incubated for a further 2 h at 37 °C with 200 µl/well of affinity-purified, HRPO-conjugated goat antimouse IgG (Boehringer-Mannheim, Dorval, Quebec) diluted 1/1,000 in PBST. The plates were washed six times in PBST, and the substrate used was 5-aminosalicylic acid at 0.8 mg/ml with 0.006% H_2O_2. The colour was allowed to develop for 30 min. The absorbance at 492 nm was measured in a Dynatech Microelisa reader (Model MR 580) using wells without monoclonal antibody as controls.

Competitive binding assays

The ELISA technique described above was modified for competitive binding assays by substituting the monoclonal and HRPO-conjugated second antibodies with HRPO-conjugated monoclonal antibodies. Color development and measurement of absorbance was as described above. The percentage competition was calculated using the formula

$$\frac{[100\,(A - n)]}{A - B}$$

where A is the optical density (OD) in the absence of competitor (i.e. unrelated monoclonal antibody conjugated with peroxidase), B is the OD in the presence of homologous antibody and n is the OD in the presence of competitive antibody [10].

Competition was regarded as any level greater than 80%. Intermediate competition was judged as between 60—80% competition, levels between 60% and −20% were considered as non-competitive, less than −20% was designated enhancement (negative competition).

Polyacrylamide gel electrophoresis (PAGE)

Sodium dodecyl sulphate (SDS) PAGE was carried out according to the semi-denaturing method of Cohen et al. [4]. In this system the stacking gel buffer was 0.125 M Tris-sulphate pH 6.6 and the running buffer Tris-borate pH 8.3 containing 0.1% SDS. Samples were diluted in stacking gel buffer with 0.5% SDS but were not boiled before application to the gel. Gels were of 7.5% acrylamide (29 : 1, acrylamide : bisacrylamide) and 0.75 mm thickness. Electrophoresis was performed in a Hoeffer Mighty Small II apparatus (Hoeffer, San Francisco, CA) at 25 mAmps.

Western blotting

After termination of electrophoresis, gels were equilibrated in transfer buffer (0.192 M glycine, 0.025 M Tris, 0.1% SDS, 20% v/v methanol) for 5 min before subjection to electrophoretic transfer to nitrocellulose. Transfer was carried out in a Hoeffer Mighty Small Transphor unit at 100 V for 30 min in transfer buffer. Following transfer the nitrocellulose was washed in distilled water and then equilibrated in 0.02 M Tris-HCl, 0.5 M NaCl, pH 7.5 (TBS). A blocking reaction was performed in 3% gelatin in TBS for 1 h on a rotary shaker. The monoclonal antibody was applied at a dilution of 1/50 in 1% gelatin in TBS and the blot incubated overnight on a rotary shaker at room temperature. After washing in TBS the reaction was amplified by incubation for 2 h with rabbit anti-mouse IgG (heavy and light chains) (Cappel) at a dilution of 1/100 in TBS with 1% gelatin. Protein A-peroxidase conjugate (Bio-Rad) was applied after washing three times in TBS at a dilution of 1/2,000 in TBS with 1% gelatin for 2 h. The colour development reagent 4-chloronaphthol was used according to the supplier's instructions (Bio-Rad).

Results

Analysis of affinity-purified gIV

When affinity-purified gIV was separated on a polyacrylamide gel, two bands were observed with apparent molecular weights of 140 K and 71 K (Fig. 1). Identical material after transfer to nitrocellulose and detection with a different monoclonal antibody against gIV than that used for purification is shown in the adjacent lane (Fig. 1B). This lane clearly shows two bands corresponding to the two stained bands in lane A (Fig. 1).

Samples of protein from each of the two bands were excised from the acrylamide gel after electrophoresis and used to immunize rabbits. The rabbit sera were then titrated by ELISA against the homologous and heterologous proteins as shown in Table 1, indicating extensive cross reactivity. Virus neutralization titres in the presence of complement are also shown.
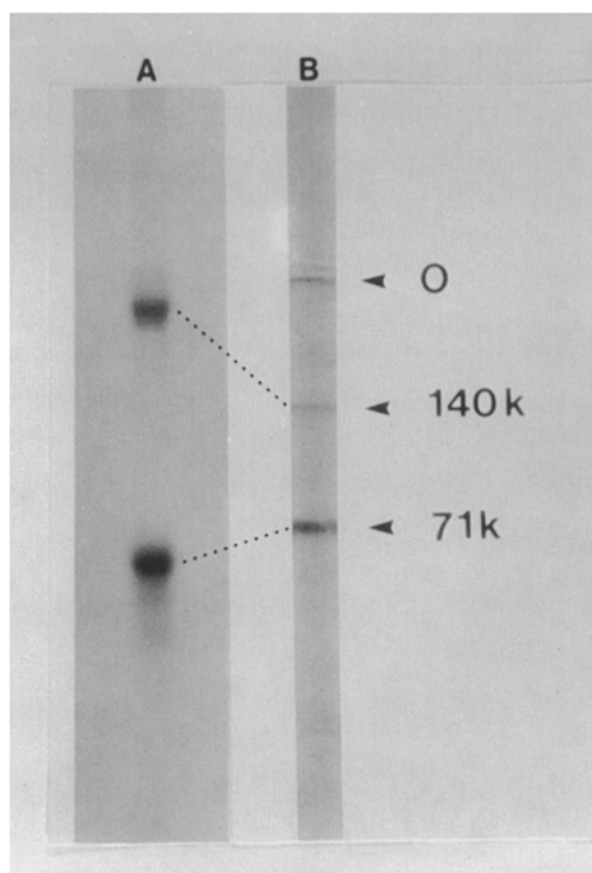

Fig. 1. Analysis of affinity-purified gIV. **A** 7.5% acrylamide gel (length, 11 cm) stained with coomassie brilliant blue, **B** Western blot of 7.5% acrylamide gel (length, 5.5 cm) using a different monoclonal antibody for detection than that used for purification. *O* Origin of electrophoresis of **B**. The different lengths of the gels account for the apparent differences in migration

Table 1. Titration of rabbit sera specific for the 71 K and 140 K species of gIV

Rabbit	Immunizing antigen	ELISA titre[a]		Neutralization titre[b]	
		71 K	140 K	no C′	C′
85-251	71 K	2×10^4	10^5	16	64
85-253	140 K	2.5×10^4	2.5×10^4	16	256
85-254	140 K	1.5×10^3	6×10^3	NT[c]	4

[a] ELISA titers were determined against either 71 K or 140 K protein adsorbed to microtitre plates, and expressed as the reciprocal of the highest dilution that still gave a reading of 0.1

[b] In the absence (no C′) or presence (C′) of complement

[c] *NT* Not tested

Characterization of gIV-specific monoclonal antibodies

Monoclonal antibodies were screened first by their ability to recognize whole virions in ELISA; using this method fifty positive clones were obtained. These clones were then tested for their capacity to mediate virus neutralization. The resulting seven neutralizing clones were subcloned and ascites fluid was prepared from selected subclones which continued to demonstrate virus neutralization. Ascites fluids from these seven monoclones were further characterized with respect to their reactivity with gIV.

Table 2. General characteristics of gIV-specific monoclonal antibodies

Monoclone[a] designation	IgG conc. (mg/ml)	ELISA[b] titer	Reactivity in	
			Western blot[c] assay	ELISA with[d] denatured gIV
2 C 8-1	9.0	10^7	—	—
2 C 8-2	10.5	10^7	—	—
3 C 1	10.5	10^7	±	—
3 E 7	10.0	10^6	—	—
4 C 1	9.0	10^7	+	—
9 D 6	9.5	10^7	+	+
10 C 2	NT[e]	10^7	+	+
136	9.5	10^7	±	—

[a] All monoclones were IgG 1 subclass and all monoclones were reactive in an immunoprecipitation assay

[b] ELISA titers were expressed as the reciprocal of the highest dilution that still gave a reading of 0.1

[c] gIV was separated by PAGE under semi-denaturing conditions before being subjected to Western blot analysis

[d] Microtiter plates were coated with denatured gIV

[e] *NT* Not tested

Table 3. Functional analysis of gIV-specific monoclonal antibodies

Monoclone designation	Neutralization titer[a]	Post-adsorption neutralization titer[a]	AbC' lysis[b]
2C8-1	30,000	0	52
2C8-2	50,000	0	8
3C1	50,000	0	22
3E7	4,096	0	67
4C1	2,048	0	56
9D6	4,096	50	54
10C2	NT[c]	0	5
136	50,000	500	7

[a] Neutralization titers were determined in the absence of complement and expressed as the reciprocal of the dilution which caused a 50% reduction of plaques relative to the virus control

[b] AbC' was expressed as the percentage lysis at an antibody dilution of 1 : 10

[c] *NT* Not tested

All of the monoclonal antibodies were able to precipitate the 71 K and 140 K forms of gIV from [^{35}S] methionine-labelled BHV-1 infected cells (not shown). Similarly, they all recognized gIV in its native state, as determined in an ELISA assay (Table 2). However, the ability of the different clones to detect semi-denatured gIV in a Western blot assay was much more variable; three clones (4C1, 9D6, and 10C2) showed a strongly positive reaction; two (3C1 and 136) were weakly positive, whereas the remaining three (2C8, 1 and 2, and 3E7) were negative. Only two monoclones, 9D6 and 10C2, were reactive with denatured gIV in an ELISA assay. Isotyping of these neutralizing monoclonal antibodies showed that they were all of the IgG subclass 1. The monoclonal antibodies were also analyzed with respect to their function (Table 3). Whereas all seven monoclonal antibodies were able to neutralize BHV-1 at very high dilutions, only two of them, 9D6, and 136, were able to prevent penetration of the virus into the cell after attachment. In contrast, the only dilution of antibody resulting in any antibody-mediated, complement lysis was 1 : 10.

Topographical analysis

In order to analyze epitopes on gIV, reciprocal competition binding assays (CBA's) were performed using all of the neutralizing monoclonal antibodies. Only one subclone of each monoclonal antibody was used since after characterization (Tables 2 and 3) the data indicated that the subclones included were not significantly different from each other.

Figure 2 shows the CBA's for 3E7 as the conjugated antibody. In order to facilitate interpretation the graphs have been split. The only antibody showing any competition with the conjugated antibody was the homologous 3E7; 4C1, 10C2, 2C8, and 3C1 all showed an absence of competition giving levels
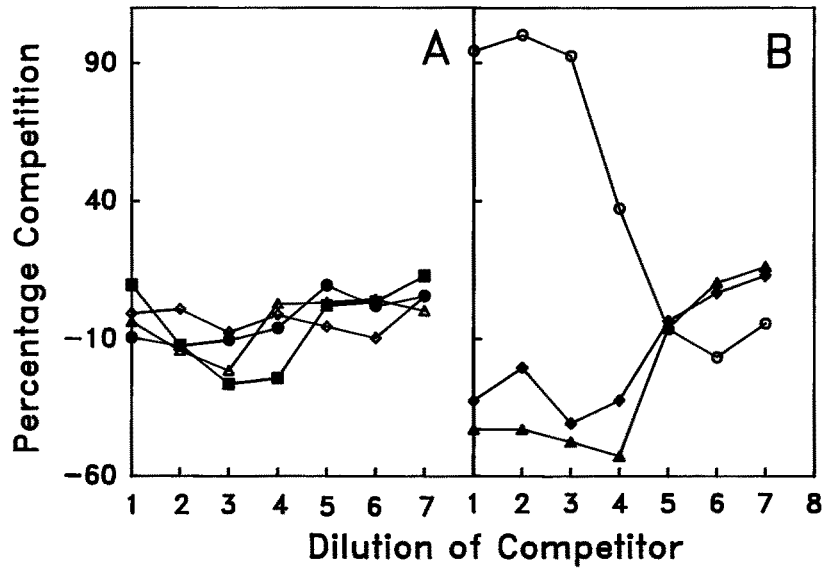
Fig. 2. Competition binding assays of various monoclonal antibodies with HRPO-conjugated 3E7 monoclonal antibody (A and B). gIV specific competitor antibodies were ■ 10C2, ● 2C8, △ 4C1, ◇ 3C1, ○ 3E7, ◆ 136, ▲ 9D6. The log 10 of the dilution is indicated on the X-axis
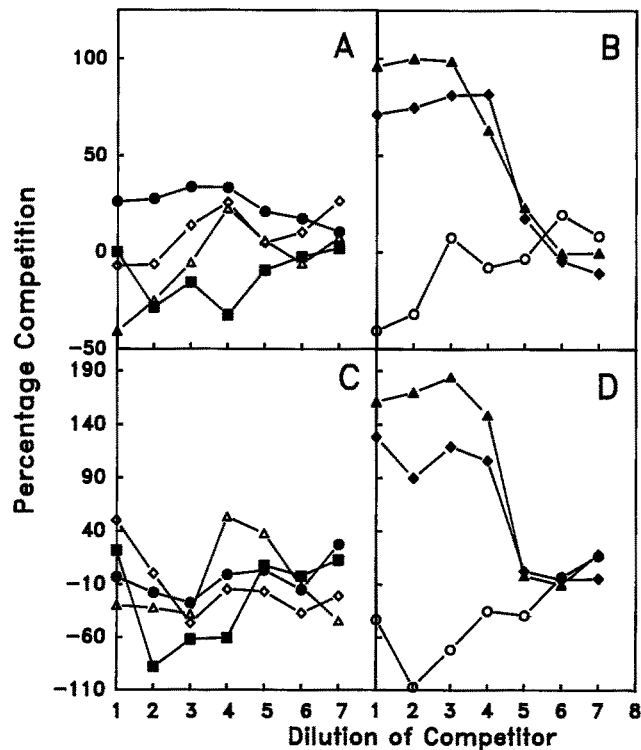

Fig. 3. Competition binding assays of antigenic domain 1 of BHV-1 gIV glycoprotein. A and B Monoclonal antibody 9D6 conjugated with HRPO, C and D monoclonal antibody 136 conjugated with HRPO, ■ 10C2, ● 2C8, △ 4C1, ◇ 3C1, ○ 3E7, ◆ 136, ▲ 9D6. The log 10 of the dilution is indicated on the X-axis
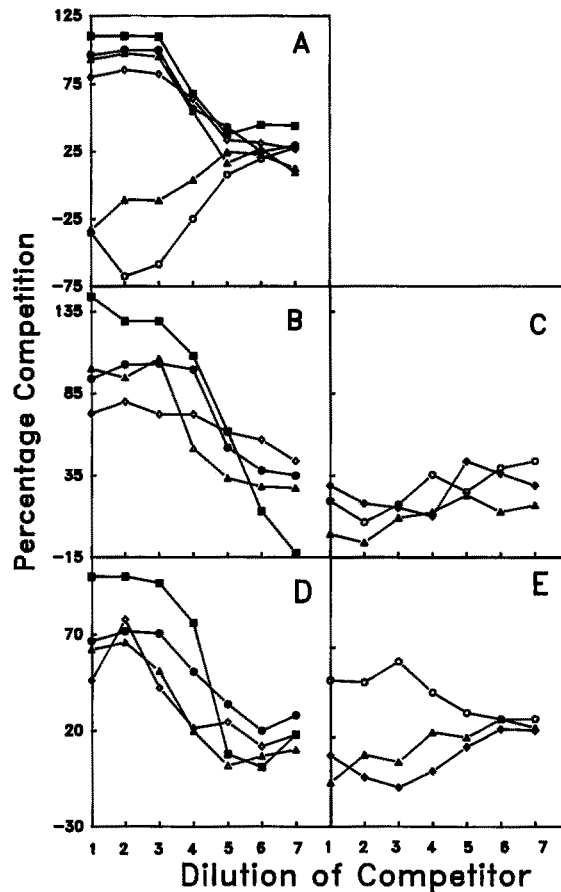
Fig. 4. Competition binding assays of antigenic domain 3. **A** 2 C 8 conjugated to HRPO, **B** and **C** 4 C 1 conjugated to HRPO, and **D** and **E** 10 C 2 conjugated to HRPO. gIV specific competition antibodies were ■ 10 C 2, ● 2 C 8, △ 4 C 1, ◇ 3 C 1, ○ 3 E 7, ▲ 9 D 6, ◆ 136. The log 10 of the dilution is indicated on the X-axis

between 10% and − 10% competition. Competing antibodies 9 D 6 and 136 showed "negative competition", i.e. enhancement.

Figure 3 shows the competition binding assays for two conjugated monoclonal antibodies, 9 D 6 and 136. These two show similar patterns, they competed with each other for antigen and show similar patterns of non-competition/ enhancement. The graphs of 9 D 6 showed the classical patterns of 100% competition and absence of competition. When 136 was the conjugate some unusual features emerged. Firstly, 9 D 6 appeared to compete with 136 more effectively than the homologous antibody, at levels in excess of 100% competition. Secondly, the binding of some other antibodies enhanced the binding of conjugated 136 giving "competition" levels as low as − 110%, by 3 E 7.

The competition patterns shown by four of the remaining monoclonal antibodies are shown in Fig. 4. When 2 C 8 was used as the conjugated antibody, 3 E 7 showed enhancement of conjugate binding. The non-competitor in this

Table 4. Summary of competitive binding assays of monoclonal antibodies directed against
BHV-1 gIV

Conjugate	Competition[a]							Antigenic domain
	10C2	4C1	2C8	3C1	3E7	136	9D6	
10C2	+ +	+	+	+				3a
4C1	+ + +	+ +	+ +	+				3b
2C8 (1+2)	+ + +	+ +	+ +	+	−			3c
3C1	+ + + +	+ + +	+ + +	+ +	+	− − −	− − −	3d
3E7					+ +	−	−	2
136	−				− −	+ +	+ + +	1a
9D6						+ +	+ +	1b

[a] Values represent degree of competition: + + + + >200%, + + + 120−200%,
+ + 80−119.9%, + 60−79.9%; or enhancement: − −60−79.9%, − − −80−119%,
− − − < −120%

graph was the 9D6 monoclonal antibody and 136 (not shown) showed a very
similar pattern. All of the other monoclonal antibodies, 4C1, 10C2, and 3C1,
competed successfully with 2C8 for antigen at levels between 80–110%. Gen-
erally similar results were found when the monoclonal antibodies were used in
competition with the conjugated 4C1 antibody. However, 3E7 did not show
enhancement with 4C1 (Fig. 4C) as it did with 2C8 (Fig. 4A). Monoclones
9D6 and 136 showed the same non-competitive pattern with 4C1 as with 2C8.
The graph (Fig. 4B) shows that 10C2 competed most strongly with 4C1 with
a maximum of 144% competition. Other antibodies (3C1, 2C8) also competed
with 4C1 but at slightly lower levels, 2C8 at the same level as the homologous
antibody, whereas 3C1 reached a maximum of 80%.

The graphs showing competition with 10C2 (Fig. 4D and E) show a broadly
similar pattern to those with the 2C8 and 4C1 conjugates. The same set of
monoclonal antibodies competed at equivalent levels, that is, the homologous
antibody 10C2 showed the maximum level of competition. The same was true
for 10C2 in competition with 9D6 and 136, which did not compete at all.
Monoclone 3E7 was also a non-competitor, although it showed slightly higher
(though not significant) levels of competition than 9D6 and 136. The results
of all of the competition binding assays are summarized in Table 4.

Discussion

Upon purification of glycoprotein gIV (71 K) by affinity chromatography and
analysis by SDS-PAGE and Western blotting, an additional polypeptide with
an apparent molecular weight of 140 K was identified. The antibody responses
of the rabbits that were immunized with the individual 71 K and 140 K poly-
peptides were evaluated by ELISA and neutralization assays, and showed ex-

tensive cross-reactivity between these two species further indicating that they are related. Judging by their apparent molecular weight, the 140 K polypeptide is probably a dimer of gIV (71 K). The dimer was not reduced to a monomer by boiling in 2-mercaptoethanol, indicating that it is not linked by disulfide bonds. However, following purification of gIV, the dimer is more prevalent than after immunoprecipitation of [^{35}S]methionine-labelled BHV-1 infected cells. Since during immunoaffinity purification of gIV the NP 40 concentration is decreased [17], the 140 K species is probably a noncovalently linked dimer of 71 K-gIV. This is in agreement with the observations reported by Marshall et al. [14] for their 77 K/150 K polypeptide.

Extremely high neutralizing antibody titres were obtained by these mono-clonal antibodies even in the absence of complement, expecially when compared with other reports [5, 15]. Only two of these antibodies, 9 D 6 and 10 C 2, recognized a continuous epitope, whereas the remaining five reacted with structural epitopes, which may explain at least some of the high neutralizing antibody titres. None of these monoclonal antibodies mediated immune lysis of virus-infected cells to any extent, whereas some monoclonal antibodies against BHV-1 glycoproteins gI and gIII showed lysis activity at dilutions greater than 1 : 10,000 [19]. This may be due to an inappropriate exposure of the critical gIV-specific epitopes on the cell membrane, making them inaccesible to either antibody or complement. Modifications or conformational changes in the gly-coproteins during virus maturation may account for such a difference in top-ological orientation.

The competition binding assays suggested the presence of three different antigenic domains: the 136, 9 D 6 group was assigned epitopes 1 a and 1 b respectively; the 3 E 7 group, antigenic domain 2 and the final group, antigenic domain 3 with four members 3 a, 3 b, 3 c, and 3 d (10 C 2, 4 C 1, 2 C 8, and 3 C 1 respectively).

Antigenic domain 2 comprised a single monoclonal antibody which showed the classical behaviour when in competition with other monoclones, i.e. 100% homologous and 0% heterologous competition. This suggests that this antigenic domain is a separate, independent epitope. Furthermore, it can be implied from the monoclonal antibody characterization data that this epitope is a discontin-uous epitope containing amino acid residues from different parts of the gly-coprotein. This was suggested by the inability of the antibody to recognize denatured antigen in ELISA or detect gIV on a Western blot. It is also important to note that 3 E 7, when acting as a competitor, showed some interaction with other antigenic domains. There appeared to be intermediate, non-reciprocal competition with 3 C 1, a member of antigenic domain 3. It also appeared to enhance the binding of 2 C 8 of domain 3 and 136 of domain 1, in a non-reciprocal manner. Interestingly, neither of these monoclonal antibodies re-cognised denatured antigen in ELISA and therefore probably recognise dis-continuous epitopes. A possible mechanism for this process is that 3 E 7 binds to domain 2, causing distortion of gIV in the envelope resulting in alterations

in other unrelated epitopes, seen as enhancement of binding. Coronavirus studies also indicate enhancement of up to 100% in the binding of specific monoclonal antibody pairs [7], and this was also shown with BHV-1 gIII [15].

Antigenic domain 1 was comprised of two monoclonal antibodies that compete with each other; they represent epitopes 1a and 1b. The differences in their levels of reactivity may be due to differences in avidity when competing for an identical site, or they may represent overlapping sites. The latter is probably the case when taken with the monoclonal antibody characterization data. The two clones reacted similarly in all tests with the exception of recognition of denatured antigen in ELISA; 9D6 was positive in this test and 136 was negative. Data from the post-adsorption neutralization assays strongly suggest that this domain is responsible for the penetration of the virus into the cell after viral attachment. Both monoclonal antibodies exhibited post-adsorption neutralizing ability although 136 was more efficient than 9D6. None of the other monoclonal antibodies showed any trace of post-adsorption neutralization. This confirms the observation that gIV is responsible for penetration of BHV-1 and indicates that antigenic domain 1 is the same as the antigenic domain IIa on gIV reported earlier [15]. Evidence from gD of HSV-1 suggesting that gD is not involved in attachment of the virion but in its uptake into the cell [3, 8] also strengthens this hypothesis. Monoclone 136 showed enhanced binding following incubation with competing monoclonal antibodies 3E7 from domain 2 and 10C2 from domain 3, which suggests that binding to these epitopes caused distortion of the glycoprotein permitting easier access for monoclonal antibody 136 to its discontinuous epitope. However, no or very slight enhancement of binding of monoclone 9D6 was observed, which is consistent with the observation that the epitope recognized by this antibody is probably continuous, since it reacted in Western blotting and it showed an ability to recognize gIV after denaturation in an ELISA (Table 2).

The final antigenic domain, domain 3, is recognized by a group of monoclonal antibodies which recognized either exactly the same epitope or overlapping epitopes on gIV. The situation in domain 3 is probably similar to that of domain 1 in that the different monoclonal antibodies represent overlapping epitopes rather than avidity differences for the same epitope. This was indicated by the antibody characterization data shown in Table 2. Each clone showed a different pattern of behaviour in Western blotting and ability to recognize denatured antigen. Clone 10C2 competed most strongly with the other members of this group. This may be due to high avidity or it may overlap with each of the other 3 epitopes. This clone also shows the maximum range of detection ability. Epitopes 3b and 3d probably overlap with each other and with 3a because they share similar characteristics, whereas epitope 3c probably only overlaps with 3a. The proposed interrelationship between these epitopes is shown in Fig. 5. Domain 3 is probably discontinuous, at least in part, as has been indicated earlier in the discussion of domains 1 and 2. This domain was involved in non-reciprocal enhancement and competition by other domains.
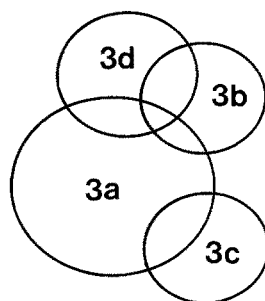
Fig. 5. Proposed interrelationship of overlapping epitopes of antigenic domain 3 on gIV of BHV-1

Furthermore, the antigen recognition data suggest that different parts of the glycoprotein brought together by secondary and/or tertiary structure contribute to the epitopes.

Taken together, the competition binding data and monoclonal antibody characteristics suggest three distinct neutralizing antigenic domains on gIV. Two of these domains, 2 and 3 are mostly discontinuous, whereas the third domain, 1, is partially continuous and responsible for virus penetration into the host cell.

Acknowledgements

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