MONOCLONAL ANTIBODIES AGAINST BOVINE HERPES VIRUS TYPE-1 (COOPER STRAIN)

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Summary. **Monoclonal antibodies (McAbs) were prepared against purified Bovine Herpesvirus Type-(BHV-1) and selected for their ability to neutralize viral infectivity. Viral purification was performed by polyethylene glycol concentration and ultracentrifugation on a 25% (w/w) sucrose cushion, and by potassium tartrate linear gradient. Out of 204 cell lines expressing antibodies to the virus, obtained in two fusions, 39 hybridomas were selected and cloned based on enzyme-linked immunosorbent assay (ELISA) reactivity. Eleven McAbs were able to neutralize BHV-1 partially or totally, with or without complement. Seven McAbs were produced as ascitic fluid, and ammonium sulfate-purified. Of these, two were able to prevent virion penetration into the cell after attachment. Three neutralizing McAbs were selected for use in immunoperoxidase tests for detecting BHV-1 in infected cell cultures.**

BHV-1, which is also known as infectious bovine rhinotracheitis virus (IBRV) is an *Alphaherpesvirinae* of the family *Herpesviridae*. BHV-1 is responsible for a variety of diseases of cattle including rhinotracheitis, infectious conjunctivitis, pustular vulvovaginitis and balanoposthitis (IPV/IPB), abortions, enteritis and encephalitis *(17)*. Like some other herpesviruses, BHV-1 can cause a long-term, latent infection in sensory ganglia and can be reactivated after stress or corticosteroid treatment *(1,19)*. BHV-1 specifies 22 structural nonglycosylated polypeptides and 11 glycosylated polypeptides *(5,30)*, and contains three major sets of envelope glycoproteins: gI, a 130-kDa dissulfide-linked 74/55-kDa heterodimer; gIII, a 180/97-kDa dimeric glycoprotein; and gIV, a 150/77-kDa dimeric glycoprotein, each of which induces the corresponding neutralizing antibodies (*11,28,31,39*). BHV-1 gI can act as a fusion protein *(13)*, gIII appears to be the major attachment protein *(27)*, and gIV is involved in virion penetration through the cell membrane *(9,37,39)* and cell fusion *(39)*.

In this report we describe the production of monoclonal antibodies against BHV-1 Cooper strain, their biological activities and, further, their use as a diagnostic tool for ELISA, immunoperoxidase and immunofluorescence. Also a comparison is made of two types of viral purification procedures.

MATERIALS AND METHODS

Cells and Virus. The Cooper strain of BHV-1 was propagated in Georgia bovine kidney (GBK) cells and purified on potassium tartrate gradient as described previously *(30)*. Alternatively, BHV-1

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was purified by precipitation with polyethylene glycol (PEG 6000) and ultracentrifugation on a 25% (w/w) sucrose cushion (personal communication Brocchi, 1992, Istituto Zooprofilattico Sperimentale Della Lombardia e Dell'Emilia, Brescia, Italia).

Electron Microscopy. Virus suspensions of each purification were placed on a grid and negatively stained with phosphotungstic acid. Electron micrographs were taken in a Zeiss EM 900 microscope at x 20000 magnification.

Production of monoclonal antibodies. Female BALB/c mice were immunized with four intraperitoneal injections of 0.5 ml purified BHV-1. The first inoculation (100 μg per mouse) was an emulsion of BHV-1 suspension containing $10^{9.5}$ $TCID_{50}$/ml in an equal volume of Freund's complete adjuvant (FCA). Two weeks later the mice were boosted with 50 μg of BHV-1 emulsified with Freund's incomplete adjuvant. This booster was repeated one week later, and the last injection consisted of 50 μg per mouse of BHV-1 in phosphate-buffered saline (PBS, pH 7.4). Three days following the last booster injection, the spleen cells from the immunized mice were fused with the murine cell line SP-2/0. The procedures of Goding, 1983 *(18)* and St. Groth & Scheidegger, 1980 *(36)* were adopted, with slight modifications. The supernatants of growing hybridomas were screened for production of BHV-1 specific antibodies by indirect ELISA and cloned by limiting dilution method. Immunoglobulin subclasses were determined by double diffusion. The selection of McAbs for characterization was based on ELISA reactivity and their ability to neutralize infectious BHV-1, with or without active guinea-pig complement. Ascitic fluids were obtained by intraperitoneal inoculation of 10^6 to 10^7 specific antibody-producing hybridoma cells into BALB/c mice that had been primed 7-10 days previously with pristane *(2,6,10,14*-tetramethylpentadecane) or FCA *(26,38)*.

Purification of antibodies. Ascites fluids were diluted 1:4 with PBS (pH 7.2) and mixed with equal volumes of saturated ammonium sulfate. The precipitate was allowed to form at 4°C for 30 min before centrifugation and dialysis against PBS (pH 7.2) *(29)*. The completion of dialysis was monitored by Nessler reactive for sulfate *(16)*.

Neutralization tests

Virus neutralization test (VN). The activity of McAbs was tested by viral titration in presence or not of a fixed dose of hybridoma supernatant. Complement-enhanced neutralization was determined by addition of guinea-pig serum at 2.5% (final concentration). A mixture of hybridoma supernatant and viral suspension preincubated for 90 min at 37°C was added to each 96-well plate seeded with GBK cells. The plates were incubated at 37°C for 3 days. Reduction of viral activity was evaluated comparing both titers 50%. McAbs were considered positive, when they reduced one or more logarithms of infectivity *(12)*.

Plaque reduction test (PRT). BHV-1 neutralizing activity of hybridoma cell cultures, ascitic fluids and ammonium sulfate-purified McAbs were tested by a standard PRT assay in 24-well plates *(3)*. Active guinea-pig complement was used in some experiments. McAbs were regarded as completely neutralizing when 80% reduction of plaques was obtained and partly neutralizing when showing a plaque reduction of at least 40% *(15,33)*.

Post-adsorption virus neutralization. GBK cells growing in 24-well plates were infected with 45-60 p.f.u./well of BHV-1 and incubated for 2h at 4°C. Then, the infected cells were washed once with PBS and undiluted ammonium sulfate-purified McAbs were added for 2h at 4°C. Finally, the McAb was removed and minimal essential medium (MEM) containing 2% FBS was added. The plates were incubated at 37°C for 48h. The monolayers were fixed in formalin and stained with crystal violet. A McAb was considered neutralizing when 50% plaque reduction, relative to the virus control, was obtained *(13,20)*.

Titration of monoclonal antibodies

Virus neutralization test. Virus neutralization was carried out by the end-point dilution procedure *(6)*. Two-fold dilutions of supernatants,

ascitic fluids and ammonium sulfate-purified McAbs, beginning at 1:10, were assayed in duplicate for the ability to neutralize 100 $TCID_{50}$ of BHV-1 in microtiter plates. In some experiments, active guinea-pig complement was added *(3,29,33)*.

ELISA. The method used was a modification of Voller *et al.* (1976) to *(43)*. Checkerboard titrations were performed previously to determine optimal dilution of purified BHV-1. Ninety-six well microtiter plates were coated with purified BHV-1 at a 1:200 dilution in PBS (pH 7.4). McAbs were added in 10-fold dilutions. HRPO-conjugated goat anti-mouse IgG was used as conjugate. The ELISA titer was determined as the reciprocal of the highest dilution of supernatant, ascitic fluid or ammonium sulfate-purified McAbs which gave a reading of at least 0.1 over the control in 492 nm filter *(39)*. As negative control a McAb specific for foot-and-mouth disease virus was used.

Immunoperoxidase test (IPT). GBK monolayers, grown on Lab-tek slides were infected with $10^{7.75}$ $TCID_{50}$/ml of BHV-1. When cytopathic effect (CPE) was observed, the inoculated cells were air dried and fixed in 25% buffered acetone for 10 min. Ammonium sulfate-purified McAbs were added in 5-fold dilution in washing buffer (PBS 7.4, 0.05% Tween 20 and EDTA 1 mM) beginning at 1:1, to determine the optimal dilutions. After 45-60 min at 37°C the slides were washed and A-protein peroxidase conjugated added. The slides were washed and the substrate (0.1 ul/ml of H_2O_2 and 1.0 mg/ml of diaminobenzidine tetrahydrochloride) was added. After washing, they were counterstained with Harri's hematoxylin (diluted 1:5 in wash buffer) for 10 min. The slides were washed in tap water and examined with a bright field microscope *(2,32,35)*. McAb specific for foot-and-mouth disease virus and non-infected monolayers were used as negative controls, were used. A polyclonal mouse anti-BHV-1 serum was used as positive control.

RESULTS

Purification of BHV-1. Table 1 shows the results of both methods of viral purification tested: with potassium tartrate gradient and concentration with PEG, and ultracentrifugation on a sucrose cshion.

TABLE 1. BHV-1 Purification.

	PEG and sucrose cushion	Potassium tartrate gradient
Bottles[a]	Roux 225 cm^2	Roller 850 cm^2
2°GBK titer[b] ($TCID_{50}$/ml)	$10^{6.5}$	$10^{7.75}$
Volume purified (ml)	2000	200
HBV-1 purified titer $TCID_{50}$/ml)	$10^{8.0}$	$10^{9.5}$
Final volume purified (ml)	12	2.2
Protein concentration[c] (mg/ml)	2.4	2.0
Electron Microscopy[d]	20% Whole virus 80% Capside	80% Whole virus 20% Capside

[a] Cell culture container used in viral production.
[b] Virus yield.
[c] Determined by the Bradford (Bio-Rad) protein assay.
[d] Transmission electron microscope (negative staining).

Electron Microscopy. Electron microscopic examinations (Table 1) confirmed that the virus obtained by potassium tartrate gradient was relatively intact. Eighty percent of the observed virions were completely enveloped, and the preparation contained no visible contamination with cell membranes (Fig. 1). The virus obtained by concentration with PEG and ultracentrifugation on a sucrose cushion (Fig.2) revealed little contamination with cell membranes, but the viral envelope was considerably damaged, and only 20% of the virions were completely enveloped. Defective particles were found in both purifications.

Panel of monoclonal antibodies. Initial screening by ELISA of the supernatants obtained from hybridomas revealed 19 clones that reacted with BHV-1 PEG concentrated (fusion 60) and 187 purified by potassium tartrate gradient (fusion 61). Nineteen were selected and cloned from fusion 60 and twenty from fusion 61 based on the highest results in ELISA. The losses on cloning efficiency were approximately 40%, resulting in nine McAbs from fusion 60: six IgG1, one IgG2a, one IgG2b and

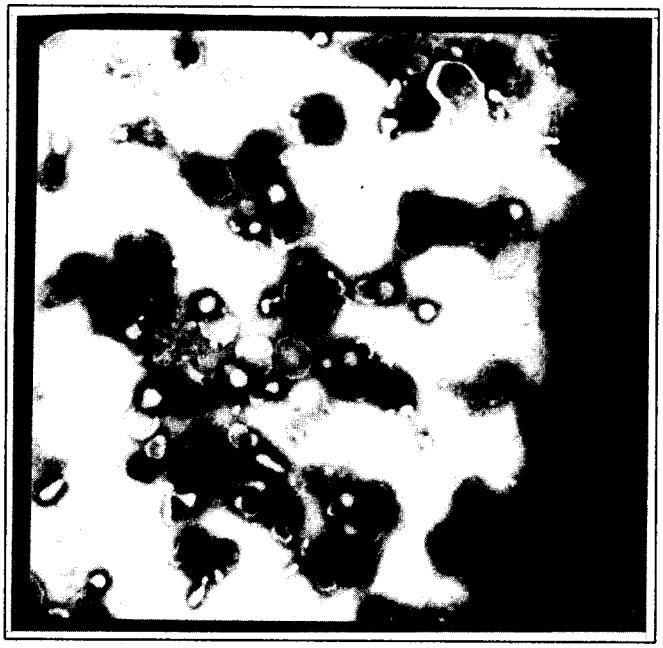

FIGURE 1. Electron micrograph of BHV-1 purified on potassium tartrate gradient (magnification: 2 x 104).

one not determined; and 14 from fusion 61: five IgG1, three IgG2a, two IgG2b, one IgG3 and three not determined.

Finally, seven McAbs (two from fusion 60 and five from fusion 61) were selected for further production of ascitic fluids and ammonium sulfate-purification based on supernatant neutralization activity (Table 2). All of them reduced the viral titer in one or more logarithms on initial screening. ELISA titers of supernatants, ascitic fluids and ammonium sulfate-purified McAbs are shown in Fig.3. Ascitic fluids and ammonium sulfate-purified McAbs were 100 to 1000 fold higher than supernatants. Two McAbs (61AF5A9 and 61BF2D8) were able to neutralize BHV-1 in aproximately 50%, preventing the virion penetration into the cell after attachment in post-adsorption VN tests, as well as reducing the plaque enlargement. Isotyping of these two neutralizing McAbs showed that they were of the IgG subclass 2a. Results obtained in VN, PRT, Post-adsorption VN, ELISA and isotypes are shown in Table 2.

Immunoperoxidase detection of BHV-1 in infected cells by monoclonal antibodies. Microscopic examination of infected cells using x10 and x25 objectives revealed peroxidase activity represented by red-brown granular deposits in the cytoplasm and nucleus using ammonium sulfate-purified McAbs 61AF5A9, 61BF2D8 and 61DH5E11 (all of them diluted 1/5). A-protein peroxidase conjugate was used 1/50. No immunoperoxidase activity was observed in negative controls.

DISCUSSION

The results of this study suggest that the various McAbs obtained are specific for glycoproteins, based on their neutralizing activities *(7, 11, 29, 31, 39, 40)*. In general three different BHV-1 glycoproteins (gI, gIII and gIV) have been identified as targets of neutralizing antibodies *(11)*.

Two McAbs (61AF5A9 and 61BF2D8) reduced the total number of plaques in approximately 50% of the control value and also reduced plaque size in post-adsorption neutralization test. These data would suggest that these McAbs are directed against gIV. They could interfere with the infection process following attachment, and reduce the plaque enlargement (only antisera against gIV significantly inhibit plaque development[*13*]) and, they had, as well, high titers in ELISA and virus neutralization *(33)*. Glycoprotein gIV is involved in virion penetration through the cell membrane *(9,37)*. There are five antigenic areas on gIV recognized by antibodies that neutralize BHV-1. In the presence of complement, a lower concentration of antibody was necessary to neutralize the virus *(28)*.

Results obtained in neutralization tests with the McAbs 60AB4H7, 60AG5E4, 61AE12F10, 61DF2H8 and 61DH5E11 suggest that they react against gI or gIII. Glycoprotein gIII is the major attachment protein *(27)* and McAbs to gIII block BHV-1 attachment. Five antigenic areas were recognized by antibodies on gIII. Four of them showed partial neutralization in the absence of comple-

TABLE 2. Characteristics of seven monoclonal antibodies McAbs to BHV-1 Cooper strain using supernatant, ascitic fluid and ammonium sulfate purified antibodies.

McAb	Isotype [1]	Plaque reduction [2]				Neutralization titer [3]						Post-adsorption neutralization *		ELISA titers [4]		
		Ascitic fluid		Purified		Supernatant		Ascitic fluid		Purified		Purified		Super-natant	Ascitic fluid	Purified
		GPCt	GPC+	GPC-	GPC+	GPC-	GPC+	GPC-	GPC+	GPC-	GPC+	GPC-	GPC+			
60AB4H7	IgG1	80%	80%	80%	80%	<10	<10	<10	<10	80	80	No	No	2 10	3 10	4 10
60AG5E4	IgG2b	40%	80%	40%	80%	<10	<10	10	20	10	20	No	No	3 10	4 10	.5 10
61AE12F10	IgG1	No	40%	No	40%	<10	<10	<10	<10	80	80	No	No	4 10	7 10	7 10
61AF5A9	IgG2a	80%	80%	80%	80%	<10	<10	20	320	640	2560	50%+	50%+	4 10	6 10	6 10
61BF2D8	IgG2a	80%	80%	80%	80%	<10	20	180	320	320	1280	50%+	50%+	4 10	7 10	6 10
61DF2H8	IgG3	80%	80%	80%	80%	<10	<10	<10	<10	10	10	No	No	2 10	4 10	3 10
61DH5E11	IgG2b	80%	80%	80%	80%	<10	<10	10	160	40	640	No	No	4 10	7 10	6 10

[1] Murine Ig class and subclass were determined by Ouchterlony double diffusion.
[2] Results expressed as per cent of PR relative to the virus control. GPC indicates guinea-pig complement. 80% indicates complete neutralization, 40% partly neutralizing and No, non reactive.
[3] VN titers of hybridoma supernatants, ascitic fluids, ammonium sulfate-purified McAbs expressed as reciprocal of that dilution giving a 50% end point.
[4] Expressed as the reciprocal of the highest diluition that still gave a reading of at least 0.1.
* Reduction of plaque enlargement.

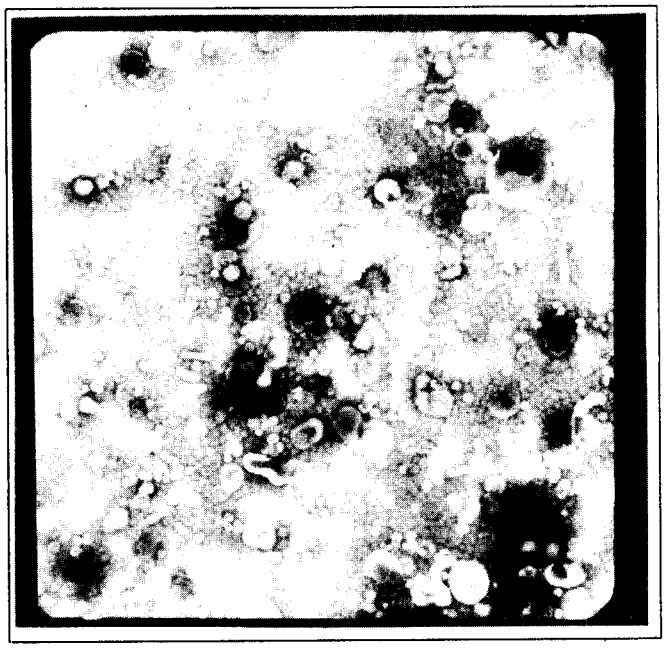

FIGURE 2.Electron micrograph of BHV-1 purified by PEG concentration and ultracentrifugation on a sucrose cushion (magnification: 2 x 104).

ment, and completely in the presence of complement. Only one antigenic area was neutralized by antibodies in the absence of complement *(28)*.

Glycoproteins gI and gIV constitute essential virion components. BHV-1 gI is important for virion penetration into cells and can act as a fusion protein *(14)*. There are four interrelated antigenic areas on gI. Three of these areas were recognized by antibodies that neutralized the virus in the absence of complement. When complement was present, a lower concentration of antibody was required to neutralize BHV-1. The fourth antigenic area was recognized by antibodies which partially neutralized without complement, and completely neutralized in the presence of complement *(29)*.

The glycoproteins specified by herpesviruses are of particular interest since they induce neutralizing antibodies. When the BHV-1 was purified by potassium tartrate gradient ultracentrifugation, the preservation of virion integrity, particularly the viral envelope, was confirmed by electron microscopy. The virus was intact and the preparation was free of host cell proteins (Fig 1). Potassium tartrate gradients have been used to purify herpesvirus giving little contamination with host cell proteins and little damage of viral envelope *(5,8,22,30)*. On the other hand, purification using PEG concentration and ultracentrifugation on a sucrose cushion resulted in disturbed structural integrity of virions, and viral envelope losses were observed (Fig 2). Defective particles were found in both purifications. These particles can be found as contaminants of a viral stock or obtained by serial passages at a high multiplicity of infection *(10)*.

An ELISA titration of cell culture supernatants, ascitic fluids and ammonium sulfate-purified McAbs revealed that the level of McAbs in ascitic fluids and ammonium sulfate-purified ascitic fluids was 100 to 1000-fold higher than in cell culture hybridoma supernatants (Fig 3). These data are in agreement with the results obtained previously *(18,39)*.

The immunoperoxidase technique is as sensitive as immunofluorescence and more sensitive than virus isolation for detection of viral

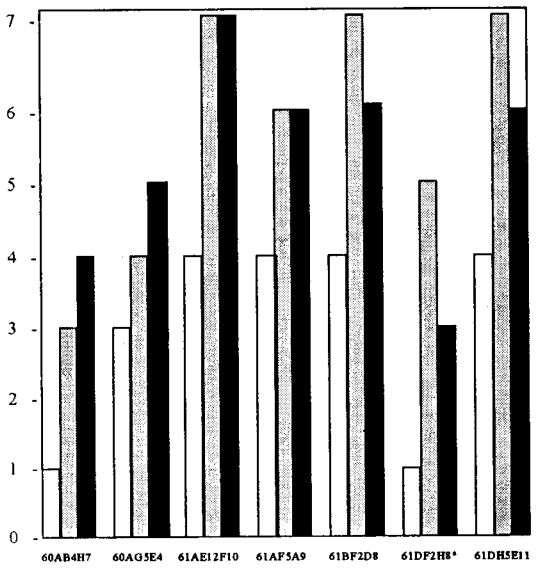

FIGURE 3. Titration of McAbs in ELISA. The ELISA titers were expressed as the reciprocal of the highest dilution that still gave a reading of at least 0.1 over the control in 492 nm. Blank column is supernatants, light shading is ascitic fluids and dark shading, ammonium sulfate-purified McAbs. *Losses on purification.

antigens in fetal tissues *(35)*. When used with hematoxylin counterstain, IPT techniques have an advantage over immunofluorescence techniques because morphologic features of cells can be studied *(34)*. Three ammonium sulfate-purified McAbs (61AF5A9, 61BF2D8 and 61DH5E11) were tested positively with infected BHV-1 cell cultures. Thus, it is suggested that these McAbs can be used as reagents in immunoperoxidase for rapid diagnosis.

Modified-live TK-negative *(23)*, gIII gene-deleted *(24)*, recombinant IBRV-foot-and-mouth disease virus *(25)*; inactivated and subunit vaccines, gI, gIII and gIV have been developed *(4,21,41,42)* to control BHV-1. The panel of McAbs prepared against gI, gIII and gIV in this study is important for producing diagnostic reagents and for further use as a possible tool to distinguish between BHV-1 infected and immunized animals in countries using subunit or genetically engineered deletion marker vaccines.

Further studies on these McAbs could focus on the following points: (i) Characterization by immunoprecipitation assay and/or by "Western" blot analysis to unequivocally identify viral glycoproteins; (ii) Characterization by reciprocal competition immunoassay to identify antigenic areas on the glycoproteins; (iii) Reactivity with other Herpesviruses to study cross-reactions, and with Brazilian BHV-1 field isolates to ensure regional diagnostic coverage of these isolates, and (iv) Use of these McAbs as a more specific and sensitive diagnostic tool in Immunoperoxidase, Immunofluorescence and ELISA tests.

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