Bovine Herpesvirus 1: Strain Comparison of Polypeptides and Identification of a Neutralization Epitope on the 90-Kilodalton Hemagglutinin

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The intracellular and structural polypeptides of the Los Angeles and Cooper 1 reference strains of bovine herpesvirus 1, together with 12 other Canadian field isolates, were analyzed by polyacrylamide gel electrophoresis. Although a few minor differences were noted among some isolates in regard to intracellular viral protein content, analysis of partly purified virus showed strikingly similar polypeptide profiles among 19 proteins with molecular masses of 14 to 145 kilodaltons (kDa). Moreover, a neutralizing monoclonal antibody produced against the Cooper 1 strain also neutralized all of the other 13 strains tested in this study and immunoprecipitated the major 90-kDa glycoprotein. A second monoclonal antibody with a high hemagglutination inhibition titer prevented hemagglutination of other strains tested and also reacted against the 90-kDa glycoprotein by immunoprecipitation, indicating that this glycoprotein is responsible for the hemagglutinating activity of the viral particle and carries an important neutralization epitope.

Bovine herpesvirus 1 (BHV-1), a significant cause of morbidity and an economically important pathogen of cattle, is associated with a variety of disease manifestations including rhinotracheitis, conjunctivitis, reproductive tract lesions, and encephalitis (2, 11). The viral particle exhibiting a typical herpesvirus morphology consists of a double-stranded DNA molecule contained in a 100-nm icosahedral capsid surrounded by an envelope. Hemagglutinating activity has been recently associated with a few Russian BHV-1 strains (5) and subsequently with several North American strains (13) for C57Br or C57BL mouse erythrocytes. BHV-1 strain P8-2 or Los Angeles has been reported to be composed of 21 to 33 structural proteins (9, 10). However, limited data are available regarding the variability of structural proteins and antigenic determinants among viral strains. Such information was analyzed in the present report on different North American BHV-1 strains.

Fourteen BHV-1 strains (infectious bovine rhinotracheitis virus) were used in this study. The Los Angeles (ATCC VR-188) and Cooper 1 (ATCC VR-864) reference strains were obtained from the American Type Culture Collection (Rockville, Md.). All others were Canadian field isolates obtained from various sources as described previously (13). Georgina bovine kidney cells kindly provided by D. W. Key, Ontario Ministry of Agriculture and Food, were used in this work and grown in equal parts of Eagle minimal essential medium and medium 199, to which were added 10% inactivated fetal calf serum and 110 μg of sodium pyruvate and 50 μg of gentamicin per ml.

For infection, newly confluent Georgina bovine kidney cell monolayers in 24-well plates (Becton Dickinson Labware, Oxnard, Calif.) were inoculated with BHV-1 strains at an input multiplicity of infection of 5: after a 1-h adsorption period, washed cells were replenished with growth medium without inactivated fetal calf serum. At 6 h postinfection, the latter medium was discarded and cells were washed and refed with 0.5 ml of Eagle minimal essential medium-medium 199 diluted 1:3 with phosphate-buffered saline and containing 10 μCi of L-[14C]alanine acid lyase (New England Nuclear Corp., Boston, Mass.) per ml. Intracellular proteins were analyzed 20 h postinfection; at that time monolayers were washed with phosphate-buffered saline, and 0.2 ml of hot (100°C) 2 × dissociating sample buffer (4.6% sodium dodecyl sulfate, 10% β-mercaptoethanol, 0.125 M Tris hydrochloride [pH 6.8]—bromophenol blue) was poured directly onto cells. Solubilized monolayers were then collected, boiled for 5 min, and centrifuged for 5 min in an Eppendorf 5412 bench centrifuge. Supernatants were collected and used for electrophoresis. For analysis of structural viral proteins, infection of Georgina bovine kidney cell monolayers was done as described above but proceeded until cytopathic effect was complete (36 h postinfection). Then viral supernatants were collected, clarified by a 30-s spin in an Eppendorf centrifuge, and ultracentrifuged through a 1-ml 25% potassium tartrate cushion for 2 h at 35,000 rpm in an SW50.1 rotor (Beckman Instruments, Inc., Fullerton, Calif.). Pellets were suspended in 150 μl of hot dissociating sample buffer, boiled for 5 min, and used for electrophoresis. Mock-infected cells were treated similarly.

Immunoprecipitation was done on labeled virus grown in a 25-cm² flask (Corning Glass Works, Corning, N.Y.) and purified as described above. Viral pellets were first solubilized in RIPA buffer (0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, 0.01 M Tris hydrochloride [pH 7.4]) for 1 h at 4°C and then centrifuged at 16,000 × g for 15 min. Mouse ascitic fluid (10 μl) was added to 150 μl of the latter supernatants, and the mixture was further incubated for 2 h at 37°C before addition of 50 μl of a 20% (wt/vol) suspension of Protein A-Sepharose CL-4B (Pharmacia;
Montréal, Québec, Canada) previously made in RIPA buffer. After another incubation period of 2 h at 37°C, we thoroughly washed the immune complexes three times in RIPA buffer before boiling them for 5 min in sample buffer. Sepharose beads were then pelleted, and the supernatant was used for electrophoresis. All electrophoreses were carried out in 8.8% sodium dodecyl sulfate-polyacrylamide gels except for immunoprecipitation studies done on 10% gels as described by Laemmli (6). Fixed gels were soaked in an Enlightening bath (New England Nuclear) for 1 h, dried, placed in Wolf cassettes (Picker Corp., Cleveland, Ohio) with X-Omat AR65 films (Eastman Kodak Co., Rochester, N.Y.), and kept at −80°C. Films were developed after 1 to 3 days.

The neutralizing and hemagglutination inhibiting (HI) monoclonal antibodies used in neutralization, HI, and immunoprecipitation studies were obtained by fusing spleen cells of BALB/c mice immunized against purified BHV-1 with mouse myeloma Ag × 63-653 cells as described previously (H. Minocha, D. Tyrell, and A. Ghram, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, S20, p. 284). Two monoclonal-antibody-producing hybridomas were selected and injected intraperitoneally into pristane-treated mice for preparation of ascitic fluids. The first monoclonal antibody selected was of an immunoglobulin G-1 (IgG-1) subclass with a neutralization titer of 1:3,000 and no HI activity, and the second belonged to the IgM class and possessed an HI titer of 1:1,280 and a neutralization titer of 1:128. Neutralization was performed in 96-well Linbro plates (Flow Laboratories, Inc., McLean, Va.) as follows. For each viral strain, 25 μl of a viral suspension containing either 25 or 50 PFU was added to wells with 25 μl of a 1:1,600 dilution of mouse ascitic fluid (IgG-1 monoclonal antibody) and allowed to react for 4 h at 4°C. Then 0.15 ml of a Georgina bovine kidney cell suspension containing 2 × 10⁵ cells per ml was added to each well, and the plates were incubated at 37°C in a 5% CO₂ atmosphere and checked for cytopathic effect after 4 days. The assay was done in quadruplicate, with viral suspensions without mouse ascitic fluid serving as controls. HI assays were performed in microtiter plates with kaolin-treated monoclonal antibodies as described previously (M. Trudel, C. Séguin, F. Nadon, G. Boulay, P. Trépanier, and G. Lussier, Vet. Microbiol., in press). Heterohemagglutinins were removed by adsorption with C57BL mouse erythrocytes. Briefly, 25 μl of a suspension containing 4 hemagglutinating units of the different BHV-1 to be tested was added to 25 μl of a 1:640 dilution of mouse ascitic fluid (IgM monoclonal antibody). The mixture was incubated overnight after which 50 μl of a 0.3% suspension of C57BL mouse erythrocytes was added. After 2 h at room temperature, the presence or absence of hemagglutinating activity was recorded.

The competitive inhibition enzyme-linked immunosorbent assay was done as described by Talbot et al. (12). We labeled monoclonal antibodies with horseradish peroxidase after evaluating the concentration of the antibodies by radial immunodiffusion against goat anti-mouse IgG or IgM (Meloy Laboratories, Springfield, Va.). The competing unlabeled monoclonal antibody was incubated with the antigen-coated plates, and the horseradish peroxidase-conjugated monoclonal antibody was then added to the wells. The reaction was developed after washing with o-phenylenediamine (0.4 mg/ml) in 0.1 M citrate buffer (pH 5). The enzymatic reaction was terminated after 30 min by adding 0.1 ml of 1 N HCl to each well. Optical densities at 492 nm were read with a Titrtek Multiscan photometer (Flow Laboratories).

The intracellular proteins of all 14 viral strains studied are depicted in Fig. 1. The overall polypeptide profiles were similar, with no major differences noted between the Los Angeles and Cooper 1 reference strains and the Canadian

FIG. 1. BHV-1 infected cell polypeptides. Samples labeled with 14C]amino acid lyate were simultaneously run in two 8.8% polyacrylamide gels with a Protean Cell Slab 1 (Bio-Rad Laboratories, Richmond, Calif.). Dots indicate virus-induced polypeptides. Arrowheads point to displaced or missing polypeptides and to a cellular contaminant (lane C). St. Molecular weight standards: phosphorylase B (92.5 kDa), ovalbumin (46 kDa), bovine carbonic anhydrase (30 kDa), cytochrome c (12.3 kDa). The BHV-1 strains are identified as follows: L.A., Los Angeles; Co-1, Cooper 1; numbers 1 to 12, 12 Canadian field isolates. C. cellular control.
field isolates. Heavily labeled major viral proteins consistently seen in all of the strains were easily identified as compared with the cellular control. A few slight variations could be observed among some strains involving either the absence or different mobilities of some minor proteins as indicated by arrowheads in Fig. 1. The presence of a major 45-kilodalton (kDa) cellular protein was also seen in all of the strains. BHV-1 structural proteins were analyzed after extracellular virus was pelleted through a 25% cushion of potassium tartrate (viral isodensity was about 30%). This approach proved quite successful in generating concentrated, intact virus preparations almost devoid of any contaminating material. Electrophoresis of such viral preparations (Fig. 2) showed that the polypeptide profiles were identical for all strains but with fewer proteins compared with the intracellular protein pattern. Major polypeptides of 145, 133, 90, 74, 64, 56, 53, 50, 46, 41, 37, 35, 33, 18, and 14 kDa were observed in all of the strains, together with minor polypeptides of 117, 105, 82, and 30 kDa. Partial purification did not succeed completely in eliminating the 45-kDa cellular protein from viruses; although its amount was greatly reduced, it was visualized both in cellular and viral lanes (Fig. 2, lane C, arrow). However, partly purified virus was devoid of most intracellular polypeptides previously seen in the 12- to 30-kDa region and which probably represented precursor degradation and cellular and viral breakdown products from larger polypeptides. Very high-molecular-weight structural proteins were also less abundant; those observed were, however, too poorly resolved to differentiate them from those in the cellular control.

Using the viral strains reported here, we then proceeded to verify the extent of neutralizing and HI capabilities of two monoclonal antibodies produced against the Cooper 1 strain. The neutralizing IgG-1 monoclonal antibody used was able to neutralize 50 PFU of the Los Angeles reference strain as well as the other 12 Canadian field isolates equally (Table 1). Similarly, the HI IgM monoclonal antibody was able to inhibit hemagglutination of 4 hemagglutinating units of all other BHV-1 strains tested.

We subsequently used these monoclonal antibodies in immunoprecipitation studies to identify the polypeptide(s) carrying the neutralization and hemagglutination epitopes. With all of the viral strains used here, the neutralizing and HI monoclonal antibodies immunoprecipitated the 90-kDa polypeptides; an example of the latter using three different strains, that is, the homologous Cooper 1 strain, the Los Angeles reference strain, and field isolate no 7, is shown in Fig. 3. Furthermore, the competitive inhibition enzyme-linked immunosorbent assay revealed that the neutralization and hemagglutination epitopes were located in two different antigenic regions since competition was seen only with the homologous monoclonal antibody and no competition was obtained between the two monoclonal antibodies with horse-

![FIG. 2. BHV-1 structural polypeptides. Electrophoresis was done as described in the legend to Fig. 1. For an explanation of the designations, see the legend to Fig. 1. The small numbers beside lane St refer to the molecular masses of individual viral polypeptides.](http://jvi.asm.org/)

<table>
<thead>
<tr>
<th>Reference strain or Canadian field isolate no.</th>
<th>Neuronalization at:</th>
<th>HI with 4 hemagglutinating units</th>
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<tr>
<td>Cooper 1</td>
<td>25 PFU</td>
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<tr>
<td>Los Angeles</td>
<td>50 PFU</td>
<td>+</td>
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<td>1</td>
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* A 1:1,000 dilution of neutralizing IgG-1 monoclonal antibody and a 1:50 dilution of an HI IgM monoclonal antibody, both produced against the Cooper 1 strain, were used in neutralization and HI assays, respectively, with each strain tested. +, Either neutralization or HI.
radish peroxidase-labeled IgG-1 were similar (data not shown), excluding the possibility that the lack of competition was due to differences in avidity.

The present report, in addition to examining BHV-1 polypeptides, also focused on possible antigenic variations, among several BHV-1 strains, of epitopes involved in neutralization and HI. As shown here with the Cooper 1 and Los Angeles reference strains and several other infectious bovine rhinotracheitis viral field isolates from Canada, a consistent intracellular polypeptide profile with little variability was observed among all strains, a finding previously reported by Misra et al. (8). However, the analysis of structural proteins after partial viral purification revealed a much clearer polypeptide profile, in which at least 19 proteins from 14 to 145 kDa were clearly identified in all of the viral strains studied. The only visible cellular contaminant appeared to be a 45-kDa protein which has also recently been reported as polypeptide C in five other herpesviruses (4). The number of polypeptides reported here is close to the 21 proteins reported by Pastoret et al. (10) for the Los Angeles strain but less than the 36 polypeptides described by Metzler et al. (7). The latter, however, directly pelleted extracellular virus, whereas we put it through a potassium tartrate cushion, probably resulting in cleaner preparations with less contaminating proteins.

Previously we reported on nine proteins (105, 90, 74, 64, 53, 50, 46, 41, and 33 kDa) against which convalescent BHV-1 rabbit serum antibodies were detected on immunoblots with the 90- and 74-kDa polypeptides being the immunodominant glycoproteins (Trudel et al., in press). Collins et al. (1) reported on four groups of neutralizing monoclonal antibodies, the most efficient recognizing a nonglycosylated protein of 115 kDa. Van Drunen Littel-Van Den Hurk et al. (14) reported that six of seven epitopes located on the 130–74–55-kDa complex and one of nine epitopes associated with the 180–91-kDa complex were involved in neutralization. A glycoprotein of 74 kDa being the main component involved in neutralization was also reported by Gregersen et al. (3). In our study, in which 14 different BHV-1 strains were used, the epitopes involved in the neutralization and HI of the Cooper 1 strain were shown to be present on all of the strains tested, indicating that no major antigenic changes could be found among isolates with regard to those specific but different neutralization and HI determinants as shown by competitive inhibition immunoassay. The importance of the neutralization epitope is also stressed by the fact that no complement was used in monitoring the neutralization of this enveloped virus. Furthermore, the epitope involved in neutralization was also shown to be present on the 90-kDa glycoprotein which was also responsible for the hemagglutinating activity of the viral particle. Thus, the demonstration that this 90-kDa glycoprotein represents the viral hemagglutinin and is an important key structure in the neutralization process suggests that this major glycoprotein would be an excellent candidate in the development of a subunit vaccine.

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LITERATURE CITED