Identification of bovine herpesvirus-1 polypeptides involved in serum neutralization

Melissa Anne Lum
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IDENTIFICATION OF BOVINE HERPESVIRUS-1 POLYPEPTIDES INVOLVED IN SERUM NEUTRALIZATION

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Identification of bovine herpesvirus-1
polypeptides involved in serum neutralization

by

Melissa Anne Lum

A Dissertation Submitted to the
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Iowa State University
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1984
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EXPLANATION OF THESIS FORMAT

This dissertation consists of an introduction, a literature review, two separate manuscripts, a general conclusion, references and acknowledgements. The Ph.D. candidate, Melissa Anne Lum, is the senior author and principal investigator for each of the manuscripts.
INTRODUCTION

Bovine herpesvirus-1 (BHV-1) is the most commonly diagnosed viral etiologic agent in bovine respiratory disease and bovine abortion (40, 42,77). Despite continued use of BHV-1 vaccines, economic losses remain severe. The efficacy of both attenuated and killed BHV-1 vaccines has been reviewed (25,51,73). In general, present BHV-1 vaccines are able to protect against disease, but none is effective in preventing infection with virulent challenge virus (51,84). Disease control is hampered by the ability of BHV-1 (wild-type or vaccine origin) to establish latent infections. In some instances, virus is shed sporadically for many years and persists through infection of new animals (91).

Nonionic detergents are thought to remove immunologically important herpesvirus glycoproteins from the virion envelope and infected cell extracts (65,96,98). A detergent-solubilized, BHV-1 infected cell extract, used as a subunit vaccine in calves, was found to be immunogenic and efficacious when administered with adjuvant. This virus-free extract induced high serum neutralizing antibody titers in all vaccinated animals; vaccinates challenged with virulent BHV-1 appeared to be protected from infection as well as disease (51). Detergent treatment of the BHV-1 virion removed thirteen polypeptides which were thought to be associated with the virion envelope (3). A detergent-soluble, virus specified glycoprotein, GVP 11 (71K dalton) was detected on the surface of BHV-1 infected cells and identified as a potential immunogen. Monoclonal antibody prepared
against GVP II was able to participate in antibody-complement mediated immunocytolysis of infected cells (56).

The objectives in this study were to identify and characterize detergent-soluble, BHV-1 specified proteins involved in inducing serum neutralizing antibody.
LITERATURE REVIEW

Bovine herpesvirus-1 (BHV-1), commonly known as the infectious bovine rhinotracheitis virus, causes a variety of disease syndromes in cattle. Infection with BHV-1 is associated primarily with respiratory disease, but reproductive, enteric, ocular, central nervous system, neonatal and dermal infections also may occur. Combined disease syndromes and persistent infections have been reported (25, 40).

Herpes simplex virus-1 proteins

Because herpes simplex viruses were the first of the herpesviruses to be analyzed extensively, their study has served as a model for the study of BHV-1. Discussion of non-BHV-1 herpesviruses, then, will be limited to herpes simplex virus-1 (HSV-1).

Forty-nine virus specified polypeptides were identified, by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), from HSV-1 infected cells (36). Thirty-three polypeptides were present in the HSV-1 virion, at least twelve of these were glycosylated (31).

Immunogenic subunits

Herpesviruses are composed, basically, of a nucleocapsid and an envelope (57). Studies comparing immune responses to nucleocapsid versus envelope antigens indicate that the immunologically important HSV-1 antigens are glycoproteins located in the virion envelope (5, 48, 98). Rabbits immunized with HSV-1 envelope antigens developed high serum
neutralizing (6,48,108) and complement fixing (5,48) antibody titers, as well as a cellular immune response (5,6); rabbits were protected from lethal challenge with HSV-1 (108). Human convalescent sera also demonstrated cellular (41) and humoral (53) immune responses against envelope antigens.

Herpes simplex virus specified glycoproteins become incorporated into host cell membranes (27,32,64,78,95). These proteins demonstrate protective activity and correspond antigenically (16,19,39,75,78,80, 82,83,87,94,98) and biochemically (95) to those present in the virion envelope. Crude, detergent-solubilized extracts from HSV-1 infected cells induce high serum neutralizing antibody titers in immunized rabbits (98), guinea pigs, hamsters and mice (44); immunized mice were protected from lethal challenge with HSV-1 (43,65).

**HSV-1 glycoproteins**

Detergent-solubilized, $^{14}$C-glucosamine-labeled, HSV-1 virions and infected cell extracts were used to form crossed immunoelectrophoretic (CIE) patterns (16,96,98,100,102). Three distinct viral glycoprotein antigens (Ags), Ag 6, Ag 8 and Ag 11 were identified consistently from these patterns (50,63,64,97,100). Polypeptide profiles of $^{14}$C-glucosamine-labeled HSV-1 virions and infected cell membrane antigens were formed using SDS-PAGE (31,32,36,95). The HSV-1 infected cell membrane glycopeptides (ICP), ICP 7, ICP 8 and ICP 18 were electrophoretically similar to the major virion glycopeptides (VPs), VP7, VP8, and VP 18, respectively (32, 94). The major virion glycopeptide, VP8.5, was resolved using a different
crosslinker in SDS-PAGE (31). The CIE Ags 6, 8 and 11 formed poly-
peptide patterns identical to those formed by the SDS-PAGE VPs 8, 18
and 7+8.5, respectively (62). An 80K glycopeptide, with affinity for
the immunoglobulin F_c region, was isolated from HSV-1 infected cell
extracts and was found to be distinct from the HSV-1 glycopeptides
mentioned above (1).

Five HSV-1 glycoproteins were recognized and designated gA, gB,
gC, gD and gE (See Table 1) with molecular weights of 119K (94), 126K
(94), 129K (31), 59K (11), and 80K (1) dalton, respectively. The gly-
coproteins gA, gB, gC and gD correspond to the virion polypeptides 8.5,
7, 8 and 18, respectively (94). Glycoprotein gE corresponds to the
80K F_c binding glycopeptide (1,58). Lactoperoxidase-catalyzed radio-
iodination experiments indicated that each of these glycoproteins was
present on the surfaces of the HSV-1 virion and HSV-1 infected cells
(1,27,58,59). Recent studies indicate that gA is a precursor to gB
and is not incorporated into virions (20,29,94).

To aid in further characterization, monospecific antibody was prepared
against each of the HSV-1 glycoproteins. A mixture of gA and gB was
prepared from infected cell extracts and separated by SDS-hydroxylapatite
chromatography; the purified antigens were used to produce monospecific
antibody in rabbits (20). Anti-gC antibody was produced by adsorbing
polyspecific sera with an HSV-1 mutant lacking gC (94), or by immunizing
rabbits with gC isolated from preparative CIE (101) or SDS-PAGE (16,75,76)
gels. Anti-gD antibody was prepared by immunizing rabbits with chromato-
graphically purified gD (11), or with gD isolated from preparative
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<td>119</td>
<td>late</td>
<td>infected cell membranes</td>
<td>precursor to gB</td>
</tr>
<tr>
<td>gB</td>
<td>126</td>
<td>late</td>
<td>virion envelope infected cell membranes</td>
<td>important for infectivity promotes membrane fusion for penetration</td>
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<td>gC</td>
<td>129</td>
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<td>59</td>
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<td>virion envelope infected cell membranes</td>
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<td>gE</td>
<td>80</td>
<td>late</td>
<td>virion envelope Fc receptor infected cell membranes</td>
<td></td>
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\(^a\) VN, virus neutralization; ADCC, antibody dependent cell mediated cytotoxicity; AbC', antibody-complement mediated cytolysis; VN(C'), complement mediated virus neutralization.

\(^b\) U\textsubscript{L}, unique long segment; U\textsubscript{S}, unique short segment.
<table>
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<tr>
<td>pgA</td>
<td>VN</td>
<td>UL</td>
<td>20,37,62,85,94</td>
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<td>ADCC</td>
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<td></td>
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<tr>
<td>pgA</td>
<td>VN</td>
<td>UL</td>
<td>20,27,37,52,59,</td>
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<tr>
<td>gA</td>
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<td>62,85,86,94</td>
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<tr>
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<td>VN</td>
<td>UL</td>
<td>12,27,31,37,52,</td>
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<td></td>
<td>ADCC</td>
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<td>59,62,85</td>
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<td>AbC'</td>
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<tr>
<td>pgD</td>
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<td>ADCC</td>
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<td></td>
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<td>pE</td>
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<td>gE1</td>
<td></td>
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<td>0.924-0.951</td>
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CIE (102), SDS-PAGE (75, 76) or Ouchterlony (103) gels. Antibody against gE was prepared by immunizing rabbits with gE purified from Fc affinity columns (1, 68).

Monospecific antibody preparations against glycoproteins gB, gC and gD neutralized infectious virus and were able to participate in antibody–complement mediated immunocytolysis as well as antibody-dependent cell mediated cytosis (62). Neutralization of infectious virus by anti-gE antibody required complement (68). Mice passively immunized with monoclonal antibody directed against glycoprotein gD or gC were protected against acute neurological disease induced by HSV-1 (18). Purified glycoprotein gD induced high titers of neutralizing antibody in mice (24), and rabbits immunized with a cloned glycoprotein gD/β-galactosidase fusion protein produced neutralizing antibody to HSV-1 (104).

**Antigenic sites**

Monoclonal antibody directed against an HSV-1 glycoprotein gC epitope was used to select neutralization resistant HSV-1 mutants. Two antigenic sites on the glycoprotein gC were discovered by comparing the mutants' patterns of resistance to neutralization by a panel of anti-gC monoclonal antibodies (34).

Seven separate HSV-1 glycoprotein gD determinants were demonstrated by comparing reactions of seven groups of monoclonal antibodies to glycoprotein gD in radioimmune precipitation and neutralization assays. Glycoprotein gD, bound to the same panel of monoclonal antibodies was digested with *Staphylococcus* V8 protease. Digest patterns revealed two
major antigenically active gD fragments that remained bound to the antibody; a 38K fragment contained three epitopes and a 12K fragment contained one epitope. Tryptic peptide analysis revealed that the 12K fragment was a subset of the 38K fragment. Two groups of monoclonal antibodies recognized the denatured form of gD, suggesting that at least two epitopes were sequential (not conformational) in nature (22). The same panel of monoclonal antibodies was used to study the in vitro translation and processing of glycoprotein gD. One group of antibodies recognized a gD epitope located on the outside of the infected cell membrane and demonstrated virus neutralizing activity. A different group of antibodies recognized an epitope located on the inside of the host cell membrane and did not demonstrate virus neutralizing activity (54).

**Glycoprotein precursors**

The synthesis and processing of the major HSV-1 glycoproteins gB, gC, gD and gE involve the production of partially glycosylated intermediate forms that demonstrate the same antigenic activity as the fully glycosylated, mature forms (38,58,61,68,94). Monospecific antisera and monoclonal antibody prepared against mature glycoproteins also react with precursor forms (12,20,61,68,71,94,106).

The HSV-1 glycoprotein gA was found to be related antigenically to gB (20,71). Pulse chase and immunoprecipitation SDS-PAGE studies demonstrated that gA was a precursor to gB (20). The precursor polypeptide pga was synthesized first, followed by gA, pgb and finally gB (20). The fully glycosylated gB molecule was found to exist as a dimer (86). The
unglycosylated form of gB, not normally isolated from infected cell extracts, was produced when HSV-1 was grown in the presence of tunicamycin (61). Immunoblot studies using the unglycosylated form of gB indicate that the immunologic specificity of gB resides in the protein rather than the carbohydrate; glycosylation appears to be necessary for intracellular transport to and expression on the surfaces of infected cells (61).

Monospecific antibody to the HSV-1 glycoprotein gC immunoprecipitates gC and its precursors, nonglycosylated gC (85K) and pgC (110K) from infected cell extracts (12,70,72,106). The precursor molecules formed tryptic peptide patterns identical to that formed by gC (11,12,21,72). In two dimensional electrophoresis, gC exhibited greater negative charge, molecular weight and heterogeneity than pgC (12). These data suggest that gC maturation does not involve proteolytic cleavage (94), but results solely from the addition of carbohydrate to the underglycosylated forms (12). This hypothesis was supported by studies monitoring the synthesis of gC in the presence of tunicamycin and the sensitivity of both pgC and gC to endoglycosylase (72) and mild alkaline hydrolysis (105,106). Results indicated that high mannose oligosaccharides were added to the growing gC polypeptide to form pgC. The N-linked, high mannose oligosaccharides were processed to complex oligosaccharides concomitantly with the addition of O-linked oligosaccharides to pgC to form the fully glycosylated gC (66,88).

The biosynthesis of HSV-1 glycoprotein gD also is characterized by multiple stages of post-translational modification (2). Methionine and arginine tryptic peptide maps of gD and its precursor pgD were shown to be identical, indicating that the maturation process did not involve
major alterations of basic polypeptide structure (64,94). Experiments with tunicamycin indicate that an oligomannosyl residue is added to a 50K core polypeptide before it is released from the polysome (21,46,72). The precursor pgD acquires sialic acid, which is added to the oligomannosyl residue, and carbohydrate to form the fully glycosylated 59K molecule, gD (11,13,31,23). As with gB, immunoblot studies indicate that the immunologic specificity of gD resides in the unglycosylated core protein (61).

Three electrophoretically distinct forms of the HSV-1 glycoprotein gE—pE, gE₁ and gE₂—were identified in HSV-1 infected cell extracts (1,68). Proteolytic digestion and pulse chase studies indicate that they may represent post-translationally modified forms of a single translation product. The precursor molecule, pE, is thought to be processed to form gE₁, which in turn is processed to form the fully glycosylated molecule gE₂ (1).

**Biological function of HSV-1 glycoproteins**

Herpes simplex virus-1 glycoproteins gB, gC, gD and gE are the major virus specified proteins incorporated into virion envelopes and plasma membranes of infected cells (1,94). All four glycoproteins serve as immunological targets for the host immune system (62,68). In HSV-1 infected cells, glycoproteins gB, gC and gD participate as targets in antibody-dependent, cell mediated cytolysis or in complement-dependent cytology (62). These same glycoproteins also may serve as target antigens for virus specified cytotoxic T-lymphocytes (7,45,89) and natural killer
cells (8). Anti-gB, anti-gC and anti-gD sera neutralize virus in the absence of complement (11,16,75,92,102), whereas virus neutralization by anti-gE sera requires complement (68). All four HSV-1 glycoproteins possess type common and type specific antigenic determinants (10,11,22,23,28,30,34,60,62,68,75,102,109,110,111).

Studies with HSV-1 glycoprotein gB temperature sensitive mutants suggest that gB is required for virus infectivity. Glycoprotein gB is thought to promote fusion between the virion envelope and host cell membrane for nucleocapsid entry into the host cell (4,20,49,52,67,86,90). Herpes simplex virus-1 mutant recombinants were used to map nucleotide sequences which specify determinants involved in syncytium formation, rate of virus entry and production of gB to the region of the genome coding for glycoprotein gB (17).

In some instances, HSV-1 mutants defective in their ability to produce glycoprotein gC induce polykaryocyte formation in infected cells (33,35,52,79). Glycoprotein gC appears to suppress virus induced cell fusion (52,81) by modifying the fusion promoting function of glycoprotein gB (17,85).

Little is known about the biological function of HSV-1 glycoprotein gD. Because of its ability to stimulate high titters of type common virus neutralizing antibody, it is thought to play an important role in the initial stages of virus infection (11,12,13,22,94). Anti-gD antibodies have been shown to block virus induced cell fusion (26).
Herpes simplex virus-1 glycoprotein gE has been shown to function as a receptor for the Fc region of rabbit IgG (1,68). It has been hypothesized (1) that the binding of IgG to gE receptors could 1) interfere with the immune cytolysis of both virions and infected cells (14,47), 2) influence expression of viral gene products in infected cells, perhaps leading to the establishment of latency or malignant transformation (15, 47,107), 3) reduce the yield of infectious progeny from HSV-1 infected cells (15), 4) influence virus induced cell fusion (1) and/or 5) contribute to immune complex deposition in vascular tissue after certain HSV-1 infections (9).

**Bovine herpesvirus-1 proteins**

Forty-eight virus specified polypeptides were identified by SDS-PAGE analysis of BHV-1 infected cells (55). Thirty-three polypeptides were found to be present in the virion (3,55) and eleven of these were glycosylated (55).

Like HSV-1, the immunologically important BHV-1 antigens are thought to be glycoproteins located in the virion envelope and infected cell membranes. Nonionic detergents have been used to remove quantitatively HSV-1 glycoproteins, with serum neutralizing activity, from virions and infected cells (5,74,93,96,98,99). Nonidet P-40 or Triton X-100 solubilized BHV-1 infected cell extracts protected vaccinated cattle from disease following challenge with virulent virus (51).

Triton X-100 quantitatively removed two major BHV-1 polypeptides, vp8 (90K) and vp13 (73K), of potential immunologic importance from the
These two polypeptides corresponded to two glycosylated BHV-1 structural polypeptides, GVP 9 (80K) and GVP 11 (71K), reported in a separate study (3,55). Monoclonal antibody prepared against GVP 11 was used 1) to detect the presence of GVP 11 on the surfaces of infected cells, and 2) to demonstrate the ability of GVP 11 to participate in antibody-complement mediated immunocytolysis of infected cells (56).
PART I. ELECTROPHORETIC PROTEIN TRANSFER APPLIED TO IMMUNOELECTROPHORESIS

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ELECTROPHORETIC PROTEIN TRANSFER APPLIED TO IMMUNOELECTROPHORESIS

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D. E. Reed

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A method for electrophoretic transfer of a crossed immunoelectrophoretic (CIE) pattern from an agarose gel onto a nitrocellulose sheet is described. The CIE pattern to be transferred was formed using Nonidet P-40 solubilized, bovine herpesvirus-1 (BHV-1) antigens and bovine hyperimmune serum. An autoradiogram of a CIE pattern, formed using $^{35}$S-methionine-labeled, NP-40 solubilized, BHV-1 induced antigens and bovine hyperimmune serum, was used as a control pattern. Both antigen and antibody were transferred to the nitrocellulose sheet. This was demonstrated by the ability of transferred antigen to react with specific antibody (detected by the addition of $^{125}$I-labeled anti-species antibody) and transferred antibody to react with both $^{35}$S-methionine-labeled antigen and $^{125}$I-labeled protein A. This method has application for determining the specificity of monospecific sera for individual arcs in CIE patterns.
INTRODUCTION

Crossed immunoelectrophoresis has been used to establish undenatured antigen reference patterns from crude, detergent solubilized antigen mixtures (5). The specificities of sera directed against one or more antigens within the pattern most commonly are tested by intermediate gel crossed immunoelectrophoresis. The results from this technique are difficult to interpret when several antigens in the reference pattern coelectrophorese and immunoprecipitate in close proximity. A method for in situ identification of serum-specific antigens in a crossed immunoelectrophoresis reference pattern has been described (2). This method requires the labeling of each serum sample with $^{125}$I, and is impractical when many samples are to be tested. The Western blot transfer method has been applied to sodium dodecylsulfate polyacrylamide gel electrophoresis polypeptide patterns (4). Transfer of polypeptide patterns to a nitrocellulose sheet allows for in situ identification of polypeptides that react with test sera. The purpose of the present study was to adapt the Western blot method for in situ identification of serum-specific, undenatured antigens in a crossed immunoelectrophoretic pattern.
• MATERIALS AND METHODS

Preparation of Reference Antigen

Monolayers (850 cm² roller bottles) of Georgia bovine kidney cells (Dr. R. F. Solarzano, University of Missouri, Columbia, MO) were infected with bovine herpesvirus-1 (BHV-1) at a multiplicity of 0.1-1 plaque-forming units (PFU)/cell. At 100% cytopathic effect (approximately 48 hours), infected cells were harvested and solubilized in 0.025 M Tris-Tricine buffer (4.3 g/l THAM, 9.8 g/l Tricine, 0.11 g/l calcium lactate and 0.2 g/l sodium azide), containing 1% (v/v) Nonidet P-40 at pH 8.6. The solubilized material was clarified by centrifugation at 100,000 x g for 1 hour, at 4°C, and the supernatant fluid was designated 'crude antigen extract' (CAE). Radiolabeled CAE was prepared in the same manner with the addition of L⁻³⁵S-methionine (500 µCi/roller bottle; 1015.7 Ci/mmol) to cell culture media 6 hours postinfection; cells were infected at a multiplicity of 5-10 PFU/cell and harvested at 100% cytopathic effect (approximately 24 hours).

Preparation of Reference Antibodies

Hyperimmune serum was prepared in calves that were given 3 weekly, intramuscular inoculations with CAE emulsified in Freund's incomplete adjuvant. Globulin was prepared by ammonium sulfate precipitation according to Campbell et al. (1), with the final resuspension in 0.025 M Tris-Tricine buffer (TT buffer, pH 8.6) containing 0.15 M NaCl.
Establishing a Crossed Immunoelectrophoresis Reference Pattern

The procedures for crossed immunoelectrophoresis were essentially the same as those used by Vestergaard (5). One percent (BioRad, standard low m\(_\text{r}\)) agarose was prepared in TT buffer containing 1% Triton X-100 and 1% polyethylene glycol 6000 (TTT buffer, pH 8.6). The TTT buffer (pH 8.6) also was present in electrophoresis chambers. First dimension electrophoresis was performed on a 20 μl sample of CAE (approximately 0.1 mg of total protein) at 10 V/cm for 90 min, at 10°C. In the second dimension, the separated antigens were electrophoresed into agarose containing 5% reference antibody, using 1.5 V/cm for 18-24 hours at 10°C. When desired, Coomassie brilliant blue R250 stain was used to visualize precipitates.

Electrophoretic Transfer of the Reference Pattern to Nitrocellulose

After immunoelectrophoresis, the gel was removed from the plastic plate and electrophoretic blot transfer was performed according to Towbin, et al. (4), with the following modifications: (1) single sheets of filter paper were placed on the cathodic side of the gel and on the anodic side of the nitrocellulose; (2) Tris-Tricine buffer, pH 11, was used as the transfer buffer; and (3) transfer was performed for a total of 3 hours, at 0.5 A for the first 30 min, 1.0 A for 2 hours and 1.5 A for the final 30 min. When desired, Coomassie brilliant blue R250 was used to detect post-transfer precipitation patterns in the gel and on the nitrocellulose sheet.
Preparation of Test Antibodies

Test antigens, against which test antibodies were formed, were prepared by line immunoelectrophoresis (3). The antigen trough contained CAE and the 1% agarose contained 20-30% of lapine serum directed against one or more antigens present in the CAE mixture. Individual precipitin lines were harvested from the gel, emulsified with Freund's incomplete adjuvant and used to vaccinate young adult rabbits; rabbits were given 3 weekly, intramuscular inoculations.

Reaction of the Nitrocellulose-Transferred Reference Pattern with Test Antibodies

After electrophoretic transfer, the nitrocellulose sheets were treated as follows:

Step 1. One hour wash in 50 ml of 0.01 M Tris-saline buffer, containing 5% bovine serum albumin (TS-BSA buffer, pH 8.6).

Step 2. Two hour incubation with 0.5 ml of test antibody in 50 ml of fresh TS-BSA buffer.

Step 3. One hour wash in 50 ml of 0.01 M Tris-saline buffer (TS buffer, pH 8.6).

Step 4. Two 30 min washes in 50 ml of 0.01 M Tris-saline buffer, containing 0.05% Nonidet P-40.

Step 5. One hour wash in 50 ml of TS buffer, pH 8.6.

Step 6. One hour incubation with 6 μCi $^{125}$I-labeled goat anti-rabbit IgG (6.95 μCi/μg) in 50 ml of TS-BSA, pH 8.6.
Step 7. Repeat steps 3 through 5.

Controls were run by substituting other reagents for test antibody in step 2 and $^{125}$I-labeled goat anti-rabbit IgG in step 6 as follows:

(1) Antibody transfer was detected by the addition of $^{35}$S-labeled CAE at step 2 and only TS-BSA at step 6, or, only TS-BSA at step 2 and 7 μCi $^{125}$I-labeled protein A (9.2 μCi/μg) at step 6.

(2) Antigen transfer was detected by the addition of rabbit anti-CAE antibody at step 2 and $^{125}$I-labeled goat anti-rabbit IgG at step 6.

(3) Nonspecific $^{125}$I-labeled goat anti-rabbit IgG reactions were detected by the addition of reference antibody, normal rabbit antibody or no antibody at step 2 and $^{125}$I-labeled goat anti-rabbit IgG at step 6.

Autoradiography

Data were analyzed by direct autoradiography at -70°C, using Kodak XAR-5 film and an intensifying screen.
RESULTS

The CIE reference pattern (Fig. 1) was transferred intact to the nitrocellulose (Fig. 2). Coomassie brilliant blue staining of the agarose gel after transfer revealed no trace of the reference pattern. Both antigen and antibody were transferred to the nitrocellulose; transferred antigens were detected by reaction with rabbit anti-CAE antibodies (Fig. 3), and transferred antibodies were detected by reaction with $^{125}\text{I}$-labeled protein A (Fig. 4) or $^{35}\text{S}$-labeled CAE (Fig. 5). An autoradiograph of a reference pattern formed using $^{35}\text{S}$-labeled CAE (Fig. 6) did not reveal any antigens additional to those found in the Coomassie brilliant blue stained reference pattern. Nonspecific reactions of the $^{125}\text{I}$-labeled goat anti-rabbit IgG with the transferred reference pattern were not apparent. Test antibody reactions with specific antigens within the transferred CIE pattern are shown in Figs. 7, 8 and 9.
Figure 1  Coomassie brilliant blue stain of the agarose reference pattern.

Figure 2  Coomassie brilliant blue stain of the nitrocellulose sheet after transfer.

Figure 3  Autoradiograph of the transferred pattern after reaction with rabbit anti-CAE, followed by $^{125}$I-labeled goat anti-rabbit IgG.

Figure 4  Autoradiograph of the transferred pattern after reaction with $^{125}$I-labeled protein A.

Figure 5  Autoradiograph of the transferred pattern after reaction with $^{35}$S-labeled CAE.

Figure 6  Autoradiograph of the agarose reference pattern formed using $^{35}$S-labeled CAE.
Figure 7  Autoradiograph of the transferred pattern after reaction with test antibody directed against a higher antigen arc, followed by $^{125}$I-labeled goat anti-rabbit IgG.

Figure 8  Autoradiograph of the transferred pattern after reaction with test antibody directed against several lower antigen arcs, followed by $^{125}$I-labeled goat anti-rabbit IgG.

Figure 9  Autoradiograph of the transferred pattern after reaction with test antibody directed against higher and lower antigen arcs followed by $^{125}$I-labeled goat anti-rabbit IgG.
DISCUSSION

The electrophoretic transfer of BHV-1 CIE patterns to nitrocellulose was successful. The dissociation of antigen-antibody complexes at high pH probably facilitated the transfer of the protein arcs out of the agarose and onto the nitrocellulose. The immunologic reactivities of antigen and antibody appeared to survive the transfer procedure, and the ability of test antibodies to react with specific antigen arcs in the transferred pattern was demonstrated. Preliminary data (not shown) for transfer at pH 6.8 and pH 3-3.5 demonstrated incomplete transfer of patterns at both pH levels and nonreactivity of the transferred pattern with protein A at the lower pH.

Potential applications for this method include (1) determining the specificity and confirming the purity of monospecific sera, and (2) identifying receptor-ligand functions among antigens. This transfer method conceivably could be adapted to all types of immunoelectrophoretic patterns.
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PART II. IDENTIFICATION OF BOVINE HERPESVIRUS-1 POLYPEPTIDES INVOLVED IN SERUM NEUTRALIZATION

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IDENTIFICATION OF BOVINE HERPESVIRUS-1 POLYPEPTIDES INVOLVED IN SERUM NEUTRALIZATION

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SUMMARY

Bovine herpesvirus-1 (infectious bovine rhinotracheitis virus) infected cell antigens were solubilized with Nonidet-P-40. The crude antigen extract was separated by reaction with bovine hyperimmune serum in line immunoelectrophoresis; individual immunoprecipitates were used to immunize rabbits. Rabbit sera possessing serum neutralizing activity were analyzed by reaction with crude antigen extract in immunoprecipitation sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. A 77-81K dalton glycopeptide and an 82-92K dalton glycopeptide appeared to be involved, separately, in inducing serum neutralizing antibody. Two glycosylated polypeptides (69-75K and 108-115K dalton) always co-precipitated and also induced serum neutralizing antibody.
INTRODUCTION

Bovine herpesvirus-1 (BHV-1), commonly known as the infectious bovine rhinotracheitis virus, is a cause of rhinotracheitis, conjunctivitis, vulvovaginitis and abortion in cattle (4) and a cause of alimentary and systemic infections in newborn calves (9).

Bovine herpesvirus-1 specific polypeptides have been identified using sodium dodecylsulfate polyacrylamide gel electrophoresis. Thirty-three polypeptides were found to be structural (1,7); eleven of these were glycosylated (7). Fifteen nonstructural viral polypeptides additionally were identified from infected cell extracts (7). An envelope glycopeptide, GVP 11 (71.5K dalton), was reported to induce antibody participating in antibody-complement mediated immunocytolysis of infected cells (8).

The purpose of the present study was to identify and characterize BHV-1 antigens involved in inducing a serum neutralizing antibody response in rabbits.
MATERIALS AND METHODS

Reference Reagents

Monolayers (850 cm$^2$ roller bottles) of Georgia bovine kidney cells (Dr. R. F. Solarzano, University of Missouri, Columbia, MO) were infected with BHV-1 (vaccine strain from Burroughs Wellcome Company, Kansas City, MO), at a multiplicity of 0.1 to 1 plaque-forming unit (PFU)/cell. At 100% cytopathic effect, infected cells were harvested and solubilized in 0.025 M Tris-Tricine buffer, containing 1% (v/v) Nonidet P-40, at pH 8.6 (TTNP-40 buffer). The solubilized material was stirred for 1 hour at 4°C, sonicated for 3-20 second bursts at 100 watts, and clarified by centrifugation at 100,000 X g for 1 hour at 4°C. The supernate was designated crude antigen extract (CAE). Radiolabeled CAE was prepared in the same manner, with some modifications. Cells were infected at a multiplicity of 10 PFU/cell and $^{35}$S-L-methionine (500 μCi/roller bottle; 400 Ci/mmol specific activity) or $^{14}$C-D-glucosamine (13 μCi/roller bottle; 60 mCi/mmol specific activity) was added to cell culture media 6 hours post-infection. Crude antigen extract control (CAE control) was prepared from uninfected Georgia bovine cells in an identical manner.

Hyperimmune serum was prepared from a calf that was inoculated with CAE emulsified in Freund's incomplete adjuvant. The calf was given 6 inoculations (3.5 mg CAE/dose) within a 12 month period. The serum was ammonium sulfate precipitated according to Campbell et al. (2), with the final resuspension in 0.025 M Tris-Tricine buffer containing 0.15 M NaCl; globulin was designated "reference antibody".
Monospecific Rabbit Sera

Crossed immunoelectrophoresis was performed as reported previously (6); the sample well contained 20 μl CAE (0.14 mg) and the 1% agarose gel contained 5% reference antibody. Groups of antigen arcs that immunoprecipitated in close proximity were cut from the gel and each group was emulsified in Freund's incomplete adjuvant and used to immunize a calf. The calf sera were ammonium sulfate precipitated as described above.

Rabbit anti-CAE serum was prepared from a rabbit given 3 weekly, intramuscular inoculations of CAE (3.5 mg/dose) emulsified in Freund's incomplete adjuvant. The rabbit serum was ammonium sulfate precipitated.

Line immunoelectrophoresis was performed according to Kröll (5). The antigen trough contained 0.5 ml (3.5 mg) of CAE and the 1% agarose gel contained 20% rabbit anti-CAE or calf antibody. Individual precipitin lines were cut from the gels, emulsified with Freund's incomplete adjuvant and used to vaccinate young adult rabbits; rabbits were given 3 weekly, intramuscular inoculations. The rabbit sera were ammonium sulfate precipitated as described above. The resulting globulins were incorporated at 20% into a 1% agarose gel and reacted with CAE (3.5 mg) in line immunoelectrophoresis. Individual immunoprecipitates were cut from the gel and used to immunize rabbits. This process was repeated until monospecific sera (sera that immunoprecipitated a single line) were obtained; seventeen rabbits and 5 calves were involved in the production of these sera. For use in immunoprecipitation SDS-PAGE and Western blot, monospecific rabbit sera were ammonium sulfate precipitated.
Serum Neutralization

The plaque-reduction serum neutralization (SN) test was used to determine rabbit anti-BHV-1 serum titers. Two-fold serum dilutions (0.3 ml) were mixed with equal amounts of MEM containing approximately 300 plaque-forming units of BHV-1. The virus/serum mixtures were incubated for 1 hour at 37°C and 0.2 ml of these mixtures were inoculated onto monolayers of Georgia bovine kidney cells in 22 mm, 12 well tissue culture plates. Cultures were incubated at 37°C for 1 hour. Inocula were removed and monolayers were washed, then overlain with 1% agarose containing MEM, 1% diethylaminoethane dextran, 2% fetal bovine serum and antibiotics. Cultures were incubated at 37°C for 48 hours before fixing and staining. Serum neutralizing antibody titers were expressed as the reciprocal of the highest serum dilution reducing the plaque count by at least 50%.

Immunoprecipitation SDS-PAGE (IP-PAGE)

Fifty μl of CAE was incubated with 100 μl of reference antibody or monospecific rabbit globulin for 4 hours at 4°C. If reference antibody was used, 10 μl of rabbit anti-bovine IgG (Cappel Laboratories, Cochranville, PA) was added and the mixture was incubated for an additional 4 hours at 4°C. One hundred μl (0.01 mg) of Protein A Sepharose CL4B (Sigma Chemical Company, St. Louis, MO) was then added and the resulting mixture was stirred every 15 minutes for 1 hour at room temperature. Sepharose beads were pelleted by centrifuging for 5 minutes at 13,000 X g, and washed 3 times with TTNP-40 buffer. After the final wash, the supernate
was removed and the beads were resuspended in 50 μl of dissolving buffer (0.2 M Tris (pH 6.8), 10% SDS, 20% glycerol and 1% bromophenol blue) and 10 μl of 2-mercaptoethanol. The mixture was placed in boiling water for 3 minutes and stored at -20°C.

Samples were electrophoresed (30 mA constant current for approximately 3.5 hours) in the presence of SDS, through a discontinuous, 10% polyacrylamide gel crosslinked with N,N'-diallyltartardiamide (DATD). Gels were dried and autoradiography was performed using Kodak XAR-5 film. Molecular weights of viral polypeptides were determined by comparison with protein standards of known molecular weights.

Western Blot

One ml of CAE (7 mg total protein) was treated with dissolving buffer and 2-mercaptoethanol, loaded into a 2.5 X 13 mm well and electrophoresed in the presence of SDS as described above. After electrophoresis, the gel was given three-10 minute washes in 0.025 M sodium phosphate buffer, pH 6.8 (transfer buffer). Electrophoretic blot transfer was performed as described previously (6) except that the pH 6.8 transfer buffer was used. The nitrocellulose sheet, containing the transferred SDS-PAGE pattern was cut into 1 cm strips and treated, with constant shaking, as follows: 1) three 15 minute washes in 10 mls of 0.01 M Tris-saline buffer, containing 0.05% Tween 20, at pH 8.6 (TS-TW20 buffer), 2) two hour incubation with 0.5 ml of globulin in 10 ml of TS-TW20 buffer, 3) repeat step 1, 4) one hour incubation with 6 μCi $^{125}$I-protein A
(6.95 μCi/μg specific activity) in 10 ml of TS-TW20 buffer, 5) repeat step 1. Data were analyzed by direct autoradiography at -70°C using Kodak XAR-5 film and an intensifying screen.
RESULTS

Serum Neutralization and Immunoprecipitation SDS-PAGE

Immunoprecipitation SDS-PAGE reference patterns were formed using reference antibody and $^{35}$S-methionine (Figure 1,B) or $^{14}$C-glucosamine-(Figure 1,A) labeled CAE. A minimum of 10 polypeptides was detected; eight of these were glycosylated.

Monospecific rabbit globulin, prepared against individual immunoprecipitates cut from line immunoelectrophoresis gels, were reacted in IP-PAGE with radiolabeled CAE. The IP-PAGE results are summarized in Table 1. Three basic polypeptide patterns were found to be associated with SN activity. Group A sera (n=4) had a geometric mean SN antibody titer (GMT) of $\geq 1:45$ (range 1:32 to $>1:64$) and precipitated a major glycopeptide with a molecular weight of 77-81K dalton (Figure 1,C and D). Group B sera (n=4) had a GMT of 1:6 (range 1:2 to 1:16) and precipitated a major glycopeptide with a molecular weight of 82-92K dalton (Figure 1, E and F). Group C sera (n=4) had a GMT of 1:6 (range 1:2 to 1:16) and precipitated 2 major glycopeptides with molecular weights of 69-75K and 108-115K dalton (Figure 1,G and H). These major glycopeptides appeared as four distinct bands when $^{35}$S-methionine-labeled CAE was reacted simultaneously with Groups A, B and C sera (not shown). One of the four serum samples in each group (A, B, or C) was selected to form its representative pattern in Figure 1.

Two minor polypeptides, 47K and 49K dalton molecular weights, co-precipitated along with the major glycopeptides. These polypeptides
Figure 1  Immunoprecipitation SDS-PAGE results. Crude antigen extract, labeled with $^{14}$C-glucosamine, was used for immunoprecipitation in lanes A, D, F and H. Crude antigen extract, labeled with $^{35}$S-methionine, was used for immunoprecipitation in lanes B, C, E, G, I and K. Polypeptides in lanes C and D were immunoprecipitated by Group A sera. Polypeptides in lanes E and F were immunoprecipitated by Group B sera. Group C sera immunoprecipitated polypeptides in lanes G and H. Crude antigen extract control, labeled with $^{35}$S-methionine, was used for immunoprecipitation in lanes J and L. Rabbit sera, produced against antigens that did not induce SN antibody, was used for immunoprecipitation in lanes K and L. Reference antibody was used for immunoprecipitation in lanes I and J.

Western blot results. Reactions of the CAE, SDS-PAGE pattern in Western blot with rabbit sera produced against CAE control, reference antibody, Group A, Group B and Group C sera are shown in lanes M, N, O, P and Q, respectively.
Table 1. Characterization of serum neutralizing monospecific rabbit sera by immunoprecipitation SDS-PAGE

<table>
<thead>
<tr>
<th>Molecular Weight (kilodalton)</th>
<th>Glycosylation</th>
<th>Group A sera</th>
<th>Group B sera</th>
<th>Group C sera</th>
</tr>
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<tbody>
<tr>
<td>131</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>108-115</td>
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<td>99-104</td>
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<td>-</td>
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<tr>
<td>82-92</td>
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<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>77-81</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>69-75</td>
<td>+</td>
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<td>-</td>
<td>+</td>
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<td>50-53</td>
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<td>-</td>
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<tr>
<td>49</td>
<td>+</td>
<td>+</td>
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<tr>
<td>47</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

^a Molecular weight values were averaged from patterns in 4 different gels.

^b Presence (+) or absence (-) of ^14C-glucosamine in each polypeptide band.

^c Geometric mean serum neutralizing antibody titer (GMT); n=4.

^d Presence (+) or absence (-) of polypeptide bands in patterns obtained after reaction of $^{35}S$-methionine-labeled CAE with Group A, B or C sera.
appeared in IP-PAGE patterns formed by reacting reference antibody with
$^{35}$S-methionine-labeled CAE control (Figure 1,J) and in IP-PAGE patterns
formed by reacting $^{35}$S-methionine-labeled CAE with rabbit sera prepared
against an immunoprecipitate that did not induce SN activity (Figure 1,
K and L). Incubation of Group A, B or C sera with $^{35}$S-methionine or
$^{14}$C-glucosamine-labeled CAE control did not precipitate any polypeptides,
nor were any precipitated when reference antibody was reacted with
$^{14}$C-glucosamine-labeled CAE control. Group C sera appeared to precipitate
a greater quantity of the 47K and 49K dalton molecular weight polypeptides,
followed by Group A, then Group B sera.

**Western Blot**

Crude antigen extract SDS-PAGE patterns were transferred electro-
phoretically to nitrocellulose. Incubation of reference antibody with
the transferred pattern gave results similar to those obtained with
IP-PAGE, however, the bands responsible for inducing SN antibody were
ill-defined (Figure 1,N). Reactions of Group A and Group B sera with
what appeared to be the 77-81K dalton and the 82-92K dalton polypeptides
(Figure 1,O and P, respectively) were detected only after prolonged
film exposure. The 69-75K and the 108-115K dalton polypeptides (pre-
cipitated by Group C sera) were not detected by this method (Figure 1,Q).
Antibody prepared against CAE control reacted with a polypeptide of
molecular weight less than 40K dalton (Figure 1,M). Western blot results
did not correlate completely with the IP-PAGE data. Differences may
reflect the ability of IP-PAGE to detect conformational antigenic
determinants, while Western blot detects primarily sequential antigenic determinants.
DISCUSSION

Four BHV-1 glycopeptides appear to be involved in inducing anti-BHV-1 SN antibody in the rabbit. The 77-81K dalton glycopeptide induced higher SN antibody titers than the 82-92K dalton glycopeptide or the two co-precipitating (69-75K and 108-115K dalton) glycopeptides, however, differences in titer may be due to differences in antigenic mass. The 69-75K and the 108-115K dalton glycopeptides may represent 1) subunits of a single protein, 2) monomer and dimer forms of a protein (similar to herpes simplex virus-1 glycoprotein gB (10)), or 3) mature and immature forms of a protein.

The weak reactions of SN positive sera in Western blot analysis suggest a binding preference for conformational determinants. The 77-81K and the 82-92K glycopeptides appear to possess linear determinants reactive with serum neutralizing antibody. These glycopeptides may be the immunogens of choice for subunit vaccines to be produced by recombinant methods. Cloned viral proteins, prepared as bacterial fusion products, may lose conformational determinants as a result of improper protein folding.

It was difficult to compare our viral polypeptides with those reported previously, since authors of these reports used different polyacrylamide gel systems. On the basis of molecular weight, two glycopeptides reported by Misra et al. (7), GVP 9 (82K) and GVP 11 (74K) appear to correspond, respectively, to the 82-92K and the 69-75K glycopeptides reported in this study. Our 77-81K glycopeptide has not
been reported previously. It does not appear to correspond to any of the glycopeptides in Misra's patterns. This may reflect the ability of DATD-linked acrylamide gels to resolve polypeptides species that comigrate in bis-linked gels (3).

Additional studies are needed to characterize the 47K and the 49K dalton polypeptides. Our data do not clarify whether they are of host or viral origin, or whether they are nonspecific reactants in IP-PAGE. They do not appear to play a major role in inducing SN antibody; rabbit sera that immunoprecipitate greater quantities of these polypeptides do not demonstrate increased SN antibody titers.
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SUMMARY AND CONCLUSIONS

This study involved the identification and characterization of detergent soluble, BHV-1 specified antigens involved in inducing serum neutralizing antibody. The nonionic detergent, Nonidet P-40, was used to solubilize BHV-1 infected cells. Monospecific sera against individual BHV-1 antigens were prepared by preparative line immunoelectrophoresis; individual immunoprecipitates were used to immunize rabbits.

A method was devised to test the monospecificity of the rabbit sera. A BHV-1 CIE pattern was formed using the crude antigen extract and bovine hyperimmune serum. The CIE pattern was transferred electrophoretically to a nitrocellulose sheet. Transferred antigen was able to react with rabbit sera (detected by $^{125}$I-labeled anti-species antibody); autoradiography revealed the antigen arc(s) for which the sera were specific.

Monospecific rabbit sera possessing anti-BHV-1 serum neutralizing activity were analyzed by reaction with crude antigen extract in immunoprecipitation SDS-PAGE and Western blot. Results indicated that a 77-81K dalton glycopeptide and an 82-92K dalton glycopeptide were involved, separately, in inducing serum neutralizing antibody. Two glycosylated polypeptides (69-75K and 108-115K dalton) always co-precipitated and also appeared to induce serum neutralizing antibody.

The following conclusions may be made:

1. The electrophoretic transfer of BHV-1 CIE patterns to nitrocellulose
was successful and useful for determining the specificity and confirming the purity of monospecific sera.

2. Four BHV-1 glycopeptides appear to be involved in inducing anti-BHV-1 serum neutralizing antibody in the rabbit.

Further research is needed in the following areas:

1. The BHV-1 specified glycoproteins profiled in these immunoprecipitation SDS-PAGE studies should be isolated and their protective activity in cattle evaluated. Results should identify the glycoprotein(s) that could be used in an effective subunit vaccine.

2. Immunologically reactive BHV-1 infected cell glycoproteins should be compared to those present in the virion to identify structural and nonstructural components. The ability of these glycoproteins to participate in antibody-dependent cytolysis of infected cells and to induce cellular immunity should be evaluated.

3. The isolation of BHV-1 mutants lacking one or more of these proteins may provide a basis for understanding the biological function of these proteins.
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